Final Report of Major Research Project

Entitled

'Estimation of Age and Longevity of Representative Vertebrate Species by Skeletochronology'

By

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CONTENT

Sr. No.	Topics	Page No.
Annexure - IX	Summary of the Project Work	**3 - 6**
I	Formation of annual growth layers in the calcified materials of freshwater fish *Labeo rohita*	**7 - 16**
II	Determination of Age and Longevity of Freshwater Fish *Salmophasia balookee* from otoliths, scales and vertebrae	**17 - 37**
III	Estimation of Age and Longevity of Road killed Indian Common Toad *Duttaphrynus melanostictus* by Skeletochronology	**38 - 52**
IV	Age and longevity of Indian garden lizard *calotes versicolor* (Daudin 1802) by skeletochronology	**53 - 70**
V	Age can estimate in Indian bird, Red vented Bulbul *Pycnonotus cafer* by Skeletochronology	**71 - 79**
VI	Occurrence of Growth Marks in the Cross section of Phalanges in the Indian black Rat, *Rattus rattus* (Lannaeus, 1758)	**80 - 88**
	Published Papers	**89**
1	Swapnali B. Lad Suresh M. Kumbar and Abhjit B. Ghadage 2014**.** Comparison of otolith, scale and vertebrae for age estimation of freshwater exotic fish *Oreochromis mossambicus*. *Indian Journal of Applied Research*, 4(6): 537-541.	**90 - 94**
2	Kumbar S. M. and S. B. Lad (2016). Estimation of age and longevity of freshwater fish *Salmophasia balookee* from otoliths, scales and vertebrae. *Journal of Environmental Biology*, 37: 943-947.	**95 - 99**
3	Suresh M. Kumbar and Swapnali B. Lad. (2017). Determination of age and longevity of road mortal Indian common toad *Duttaphrynus melanostictus* by skeletochronology. *Russian Journal of Herpetology,* 24(3): 217-222.	**100 - 105**
4	Suresh M. Kumbar (2017). Age and longevity of Indian garden lizard *calotes versicolor* (Daudin 1802) by skeletochronology. *Russian Journal of Herpetology.* (in Press)	
5	Suresh M. Kumbar (2017). Occurrence of Growth Marks in the Cross section of Phalanges in the Indian black Rat, *Rattus rattus* (Lannaeus, 1758). *Current Science*. (Commutation)	
6	S. M. Kumbar., S. B. Lad & J. A. Kumbar (2017). Formation of annual growth layers in the freshwater fish *Labeo rohita*, Southern India. *Iranian Journal of Ichthyology*. (Communication)	

Annexure – IX

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PROFORMA FOR SUBMISSION OF INFORMATION AT THE TIME OF SENDING THE FINAL REPORT OF THE WORK DONE ON THE PROJECT

1. Title of the Project	: 'Estimation of Age and Longevity of Representative Vertebrate Species by Skeletochronology'
2. Name and address of the Principal Investigator	: **Dr. Suresh M. Kumbar,** House No. 6, MAHADA Colony Palus, Tal. Palus, Dist, Sangli, Maharashtra, India, Pin. 416 310, E-mail: smkumbar@rediffmail.com
3. Name and Address of the Institution	: Department of Zoology, Arts, Commerce and Science College Palus, Dist. Sangli, Maharashtra, 416 310
4. UGC Approval letter No. & Date	: F. No. 42-619/2013 (SR); dated: 25/03/2013
5. Date of Implementation	: 01/04/2013
6. Tenure of the project	: Four Years (01/04/2013 to 31/03/2017)
7. Total Grant Allocated	: Rs. 12,30,800.00
8. Total Grant Received	: **Rs. 8,26,800.00**
9. Final Expenditure	: **Rs. 8,41,038.00**
10. Title of the Project	: 'Estimation of Age and Longevity of Representative Vertebrate Species by Skeletochronology'
11. Objectives of the project	: Following are the objectives,

a) pattern of growth marks formation in the scales, otolths and vertebrae in fishes and in phalangeal bones in amphibians, reptiles, birds and mammalian representative species.

b) applicability and reliability of skeletochronological technique for determination of age in representative vertebrate species.

c) age compositions of a population and similarities or variability's if any between populations of the same species inhabiting different locations.

d) confirmation of formation of growth marks whether they are annual or not by conducting long term experiments.

12. Whether objectives were achieved: **Yes**, Detailed report enclosed as **Annexure-I** (give details)

13. **Achievements from the Project:** Skeletochronology is one of the best techniques for assessment of age and longevity in amphibians and reptiles due to its accuracy, reliability and applicability to the live samples. Most of the skeletochronological studies on vertebrate species emerge from temperate area; corresponding studies on tropical vertebrate species are very little. In vertebrates, annual growth layers in bones serves as indices for determining the age and longevity of individual animal (s). Therefore, the present work was initiated to study the age and longevity of representative vertebrate species inhabiting the tropical climate of Southern India by using skeletochronological method. From this project work four research papers have been published in UGC Approved peer reviewed National and International journals and also research findings have been presented in four National and International conferences. Project fellow has awarded M. Phil. degree from the Shivaji University, Kolhapur.

Sr. No.	Title of the presented Paper	Name of Conference	Organized Department	Date	Level
1.	Determination of age structure of freshwater fish *O. vigorsii* by comparison of scales, otoliths and vertebrate ring counts	ICCMBSD-2015	Department of Zoology, S. G. M. College, Karad.	22-23/04/2015	International
2.	Age and longevity study of road mortally Indian common toad *Duttaphrynus melanostictus* (Schneider, 1799) by skeletochronology"	NCBBM-2016	Department of Zoology, Shivaji University, Kolhapur	15 -16/01/ 2016	National
3.	Comparison of otolith, scale & vertebra for the age determination in freshwater fish *S. balookee* from the Krishna River	XXVI National Symposium on Chronobiology	Department of Zoology, Mysore University, Mysore	02-03/06/2016	National
4.	Occurrence of growth marks in the phalanges of Indian black rat *Rattus rattus*	South Asian Small Mammal conservation Conference	SMCRF, Kathmandu, Nepal	27-29 / 08/2017	International

14. **SUMMARY OF THE FINDINGS**: (IN 500 WORDS)

The results of the present investigation contribute in following scientific findings in the field of vertebrate gerontology,

1. Long term experimental study conducted in the agricultural pond at Gavan village, Sangli District, Maharashtra has confirmed that the formation of growth marks are annual in the freshwater fish *Labeo rohita* inhabiting southern India therefore, they can be regarded as 'year rings' for assessing the age of individual animal.

2. Comparison of otoliths, scales and vertebrae ring counts for assessment of age and longevity of freshwater fish *Salmophasia balookee*. One to five growth rings consisting of growth zones and lines of arrested growths (LAGs) were noticed in different body sized fishes. The percent agreement between otolith and scale ages were 88.81% and otolith and vertebrae ages was 99.25%. Results indicate that otoliths and vertebrae are most suitable aging materials compared to those of scales in *S. balookee*.

3. Ninety three road mortal Indian common toad, *Duttaphrynus melanostictus* were used for assessment of age and longevity by skeletochronology. This is the first time road mortal toads were used for age structure study. Among the toads studied, 25.86% showed no LAGs, 30.10% one LAG each, 23.65% two LAGs, 13.98% three LAGs, 5.38% four LAGs and 1.07% of toads exhibited nine LAGs in their phalangeal histology. This toad may live for 10 years in nature.

4. Age structure of 40 individuals (22 males and 18 females) of Indian garden lizard *Calotes versicolor* inhabiting southern India was determined by skeletochronology. Average snout vent length (SVL) was 9.49 ± 1.74 cm and 8.35 ± 1.07 cm, whereas the median age was 2.77 ± 1.31 (range = 2 - 5) for males and females respectively. No statistically significant differences were noticed in body mass and SVL between the sexes. However, there was a positive correlation between body mass and SVL (r = 0.86). The maximum longevity of this lizard is 5 - 6 years in natural population.

5. Bone growth marks consisting of growth zones and lines of arrested growth (LAGs) are detected in the cross sections of phalanges of Red vented Bulbul *(Pycnonotus cafer)* studied from the first time. One to five growth marks are noticed in the cross section of red vented bulbul.

6. Skeletochronological technique was applied first time for determination of age in tropical rodent species. Bone growth marks consisting of growth zones and lines of arrested growth (LAGs) are detected in the cross sections of phalanges in Indian rodent species *Rattus rattus*. One to five growth marks are noticed in the cross section of phalanges indicating this species live for a maximum of five years.

15. Contribution to the Society (Give details) : The project funding has been extremely useful for capacity building of students and research scholars in the area of vertebrate ecology, demography and life history.

16. Whether any Ph.D. enrolled / produced out of the project : **Yes,** Project Fellow Awarded M. Phil. degree from the Shivaji University, Kolhapur and now Registered for Ph. D. Degree

17. No. of publications out of the Project (please attach) : Four papers have been published

1. Swapnali B. Lad **Suresh M. Kumbar** and Abhjit B. Ghadage (2014). Comparison of otolith, scale and vertebrae for age estimation of freshwater exotic fish *Oreochromis mossambicus*. *Indian Journal of Applied Research*, 4(6): 537-541.

2. Kumbar S. M. and S. B. Lad (2016). Estimation of age and longevity of freshwater fish *Salmophasia balookee* from otoliths, scales and vertebrae. *Journal of Environmental Biology*. 37: 943-947.

3. Suresh M. Kumbar and Swapnali B. Lad. (2017). Determination of age and longevity of road mortal Indian common toad *Duttaphrynus melanostictus* by skeletochronology. *Russian Journal of Herpetology.* 24(3): 217-222.

4. Suresh M. Kumbar (2017). Age and longevity of Indian garden lizard *Calotes versicolor* (Daudin 1802) by skeletochronology. *Russian Journal of Herpetology.* (in Press)

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6

Topic - I

Formation of Annual Growth Layers in the Freshwater Fish *Labeo rohita,* Southern India

Abstract: Fingerlings of the freshwater fish *Labeo rohita* were maintained in the agricultural pond (size 33 x 33 x 3 m) at Gavan village, district Sangli, Maharashtra, India to know the periodicity of formation of growth marks in the calcified materials by skeletochronology. Fingerlings weighing approximately 1g and body size 1.20 cm were released in June 2015 and maintained for a year in the agricultural pond under natural conditions. All fish were collected back in July 2016 by using cast net with the help of local fisherman. Among the collection, 50 fish with different body size range (18 – 39 cm) were used for age determination. There was a significant increase in body mass, body size, and otolith weight in each fish. All these fish showed one LAG each in scales, otoliths and vertebrae sections, suggesting that the formation of growth marks is annual in this fish.

Key words: Growth marks, annual, southern region, fish, *Labeo rohita*

Introduction: Studies on growth, age, and maturity of commercially important fishes provide baseline information that typically assists with the initial reorganization and delineation of geographic regions that are representative of individual stocks (Klevezal, 1996; Pawson and Jennings, 1996) and is an essential prerequisite for successful stock identification (Griffiths, 1997). The age and growth studies of major carps of India namely, *Catla catla*, *Cirrhina mrigala* and *Labeo rohita* have been investigated in detail (Jhingran, 1957; Khan and Siddiqui, 1973). The applicability and the accuracy of age determination studies depend upon the periodicity of formation of growth marks (Jhingran, 1957; Mir et al., 2013). However, very few experimental studies have established that the formation of growth marks in scales is annual; therefore, they can be regarded as 'year rings' for the estimation of age (Menon, 1986; Johal and Tandon, 1992; Tandon and Johal, 1983a). These studies are limited to north-east region of India where the variation in annual mean temperature does exceed more than 25°C. Similar type of work is totally lacking in southern region of India, where annual mean temperature variation does not exceed 10 °C. It is not much clear that formation of growth mark is annual or not and which factors will be responsible for the formation of growth marks in the southern region freshwater fishes. To address this question, we studied the periodicity in the formation of growth marks in the freshwater fish *L. rohita* inhabiting Sangli, Southern India by skeletochronology.

Material and Methods: Agricultural pond (size 33 x 33 x 3 m) was constructed at Gavan village (17' 03^0 N and 74' 6^0 E), district Sangli, India under the Maharashtra Government Agricultural Development Scheme. Gavan is a small village located 45 km east to Sangli, which receives average annual rainfall of 450 mm from South West monsoon and it has an average elevation of 560 m asl. About 1000 fingerlings of *L. rohita* (SVL = 1.20 cm) were introduced into the agricultural pond in June 2015 and maintained for a year under the natural conditions. Fish were

fed on natural and artificial foods *ad libitum* regularly. Fish were collected back in the month of July 2016 by using cast net with the help of local fisherman. From this collection, different body sized (range 18 – 39 cm) fish (n = 50) were selected for age determination study. They were brought to the laboratory where body mass (to the nearest 0.01g) and body size (measured to the nearest cm using a thread) of each fish were recorded. Simultaneously lateral line scales, otoliths and central vertebrae were collected and fixed in 10% formalin solution for further studies. Formalin fixed scales of each specimen was cleaned in water by rubbing gently with fingers. Cleaned scale was placed in between two clean slides. The slides were tied with rubber band on either side and observed under binocular microscope (Magnus MSZ-BI) for enumerating the ring counts present on the scales and photographed by using a digital camera (ABBOT DEC- 2000). The otoliths were washed in water and cleaned from all extraneous tissue and weighed to the nearest 0.001 mg. The otoliths were then immersed in 50% glycerol and observed under a binocular microscope. The growth rings were clearly visible as alternate opaque and translucent zones that were enumerated. The central vertebrae (5th and 10^{th}) of each fish were cleaned, washed in water for 1 h and decalcified with 5% nitric acid. Decalcified vertebrae were washed under running tap water for 24 h to remove the traces of formaldehyde and nitric acid and preserved in 70% alcohol. These vertebrae were embedded in paraffin wax and sectioned (10 μm thickness) by using a rotary microtome (Model GE-70). Mid-diaphyseal sections were stained with Harris haematoxyline and observed under compound microscope for enumeration of growth marks. The relationship if any, between body size and body mass, body size and otolith weight was determined by calculating the correlation coefficient '*r*' by Karl Pearson's method (Zar, 1984).

Results: At the beginning of the experiment, small sized (approximately 1 g; size 1.20 cm) fingerlings were released and maintained up to one year in the natural agriculture pond under regular feeding (Fig. 1A). Fifty different body sized (range 18 – 39 cm) fish were selected from the stock in July 2016. The body mass and size and the otolith mass were increased significantly compared to the corresponding values at the beginning of the experiment (Table 1). All these fishes showed one LAG (Lines of Arrested Growth) in scales, otolith and vertebrae sections (Table 1; Fig. 1C-E). There was a high degree of positive correlation between body mass and body size (r = 0.87), body size and otolith mass (0.82).

Discussion: The freshwater fish *L. rohita* is widely distributed in peninsular India, and is one of the highly consuming commercial food fish in India (Jayaram, 2010). Generally physiological processes of cold blooded animals are regulated by the temperature of the water in which they live. Annual cyclicity in calcified materials leaves growth marks that indicate the number of osteogenic cycles experienced, and indirectly the age of the individual. Although the age and growth of Indian major carps inhabiting north-east region of India have been investigated in detail (Khan and Siddiqui, 1973; Mir et al., 2013; Jhingran, 1959; Natarajan and Jhingran, 1963; Bhatt and Jahan, 2015) such type of work is totally scanty in northern fishes (Seshappa, 1999). In the present study, 50 different body sized (fish of representative sizes from the stock) fishes were selected after one year and all these fishes showed one LAG each in their scale, otolith and vertebrae sections which may be formed during the lapsed period, assuming that one LAG is laid down per year. The results suggest for the first time that the formation of LAG is annual in southern Indian freshwater fishes. The fact that many of the southern Indian freshwater fishes exhibit marked seasonality in the growth rate, and reproductive activity (Patil and Saidapur, 1989; Sharma et al., 2014) suggests that skeletal material (scales, otolith and vertebrae) growth is

a cyclical phenomenon leading to the formation of LAGs. Although, gonado-somatic-index (GSI) values increase from May - September, which coincides with the onset of monsoon rains and breeding activity of the fish; from October onwards there is a decrease in GSI, and they attain their maximal values in August (Sharma et al., 2014; Sarkar, 2010; Chakrabarti and Chatterjee, 2014; Roy and Mandal, 2015). Therefore, in this fish, the LAG(s) may be laid down between May - September when the body growth is almost ceased coinciding with the wet season of the year. From January onwards when GSI masses begin to restore, the next osteogenic cycle may set in. Similarly, annual rings found in the scales of *Puntius sarana* from river Ghaggar in Rajasthan and in Sukhna lake in Punjab, formed during March-May owing to spawning stress (Tandon and Johal, 1983a). The work of *Catla catla* from Harike and Gobindsagar showed the formation of the annuli in June-July coinciding with the commencement of spawning and the beginning of the southwest monsoon (Johal, and Tandon, 1992). Therefore, skeletochronology (enumerating the presence of growth marks in the cross sections of vertebrae sections) may be used to estimate the age and longevity of *L. rohita* and perhaps other tropical freshwater fishes. Furthermore, high degree of correlation between body mass and size and body size and otolith weight indicates that body size analysis is also one of the reliable techniques for assessment of age in this fish. In conclusion, our study suggests that LAGs are formed annually in the scales, otoliths and vertebrae sections of freshwater fish *L. rohita* inhabiting southern region of India and therefore could be regarded as annual rings for estimating the age of southern Indian region freshwater fishes.

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Table1. Table showing the changes in body mass and size, otolith mass, and number of LAG in the freshwater fish *Labeo rohita* collected from the agriculture pond after one year. (Values in mean ± SD).

Sl. No.	No. of fish	Body Mass (g)	Body Size (cm)	Otolith Mass (mg)	Growth Layer
Initial (June 2015)	1000	1.0	1.20	--	0 LAG
Final (June 2016)	50	267.167 ± 163.36	25.052 ± 7.169	16.02 ± 7.058	1 LAG

Explanations to Plate

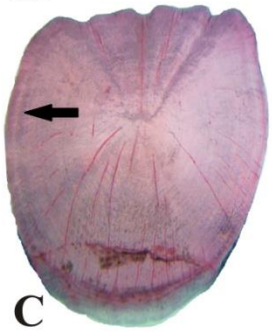
Fig. 1A - E: A: Fish maintained agricultural pond. **B**: One year old fish *Labeo rohita*, **C*:*** one LAG in the scale, **D**: otolith; **E:** vertebral cross section of the same fish; Scale line = 100 µm.

Abbreviation: Arrow = Lines of Arrested Growth (LAG).

PLATE : 1

A

B



C

D

E

Topic - II

Estimation of Age and Longevity of Freshwater Fish *Salmophasia balookee* from Otoliths, Scales and Vertebrae

Abstract: Age and longevity of freshwater fish *Salmophasia balookee* was assessed by comparing otoliths, scales and vertebrae ring counts. One to five growth rings consisting of growth zones and lines of arrested growths (LAGs) were noticed in different body sized fishes. Among the fishes (N = 134) studied, 6.72% were in the first year, 23.13% in second year, 44.03% in third year, 20.15% in fourth year and 5.97% fishes in the fifth year of growth. Precision study shows that scale ring counts (SRC) was under estimated the age compared to the otolith ring counts (ORC) and vertebrae ring counts (VRC). The percent agreement between otolith and scale ages were 88.81% and otolith and vertebrae ages was 99.25%. The results of the present study indicate that otoliths and vertebrae are most suitable aging materials compared to those of scales in *S. balookee*. This fish may live for five years in nature.

Key words: Age validation, comparison, longevity, *Salmophasia balookee*

Introduction: Studies on age and longevity provide important demographic parameters to analyze and assess the fish populations (Maceina and Sammons, 2006). However, obtaining accurate age information is crucial to the precise understanding of these metrics (Campana, 2001). Age has been determined in tropical freshwater fishes through annual increments in calcified structures like, scales, spines, vertebrae, and otoliths (Khan *et al.*, 2015). Scales are most widely used for determination of age in earlier studies, due to the fact that their removal is non-lethal, easiest to collect, and prepare (Braaten *et al.,* 1999; Kanwal and Pathani, 2011; Khan *et al.*, 2015); however, this method might not reveal the true age of slow growing and older fishes (Dua and Kumar, 2006; Kanwal and Pathani, 2011; Ujjania *et al*., 2013). Also, Khan *et al.* (2015) found that the annual rings present in scales in *H. molitrix* are not clear as compared to other structures. Indistinctness of annuli at the outer edges of scales makes them unreadable in older fishes (Akombo *et al.,* 2015). Hence, other alternative calcified materials like, otoliths, spines, opercular bones and vertebrae are used in these years as the annuli are easily recognizable even in older fish than scales (Casselman, 1990). Otoliths have several advantages for estimation of age as they are not subjected to resorption their growth is acellular rather than by ossification and also otolith annuli are more distinct and easy to enumerate, even in the older fishes (Secor *et al.,* 1995; Hoxmeier *et al.,* 2001). Phelps *et al.* (2007) reported that otoliths are to be metabolically inert and thus do not reflect physiological changes that may occur throughout the life of fish. Otoliths are grown continuously and form annuli even as body growth slows and asymptotic length is reached, and annuli reasbsorption does not appear to occur during periods of food limitation or stress (De-Vries and Frie, 1996; Colombo *et al.,* 2010). Hence, many studies have employed otoliths for estimating age in fishes (David and Pancharatna, 2003; Weyl and Booth, 2008; Colombo *et al*., 2010; Khan *et al.,* 2011). Even, vertebrae are also best calcified

material for aging in fishes (Khan et al., 2011b; Bahuguna, 2013). Therefore, selection of precise calcified materials for accurate aging of fishes is more challenging for fishery researcher. Krishna River harbours a rich diversity of fishes leading to globally threatened and endemic fishes. *S. balookee* is one of the most commercial and popular food fish in this region. In the present day it is threatened by anthropogenic stressors such as accumulation of industrial, agricultural effluents, domestic organic wastes and non-degradable plastic materials owing to tourism activities. Besides, unscientific practice for collection of fishes has been increased to meet high market demand. Since last decade sand mining and bricks manufacturing activities flourish alarmingly along some stretches of the river system. If the present trend is continued, the adverse conditions might lead to the loss of most of fish fauna of Krishna River. The proper demographic and population dynamic study is necessary for protection and conservation of this species in the river system. There is no single report on aging of *S. balookee* inhabiting the Krishna River. Therefore, present investigation is undertaken to estimate age, longevity and to evaluate and select the most reliable calcified material from scales, otoliths and vertebrae for accurate aging of *S. balookee.*

Materials and Methods: Freshwater fish *S. balookee* (*N* = 134) were collected from the Krishna River, Sangli District (17^0 09' N & 74^0 45' E), Maharashtra, Southern India with the help of local fisherman and also purchased from the local fish market in the year 2014. Fishes were brought to the laboratory where body length (BL) from the tip of snout to the longest caudal fin lobe (in cm) and body weight (BW) by using single pan balance nearest to 0.01gm of each fish were recorded. Simultaneously scales, otoliths and vertebrae of each fish was collected for comparative studies.

Scale study: Lateral line scales were collected with the help of pointed forceps, cleaned in water by rubbing through the fingers then fixed in 10% formalin solution for 24 hours and then washed

in water for 2 hours. Scale was kept in between two clean slides and slides were tied with rubber band on either side and observed under binocular microscope (Magnus MSZ-BI) for enumerating the number of growth rings present on the scales, and then photographed with digital camera (ABBOT DEC2000). **Otolith Study:** Otoliths were collected by making an incision on the dorsal side of the head, to expose the brain on either side of which the otic capsules are located. The sagittal otoliths were removed from the otic capsules by opening the otic bulla. Both sagittae were retrieved intact from each specimen, washed in water and cleaned from all extraneous tissue. Then, each otolith was weighed to the nearest 0.001 mg and the diameter of otolith was measured to the nearest 0.01 mm using a caliper rule (Newman *et al.,* 2000). Otoliths were immersed in 50% glycerol and observed under binocular microscope. Growth rings were clearly visible as alternate opaque and translucent zones that were enumerated. **Vertebrae study:** Central 5-10 vertebrae of each fish were excised, cleaned and fixed in 10% formalin solution for 24 hours, then washed in running water for one hour, later decalcified with 10% nitric acid. Decalcified vertebrae were washed in running water for 24 hours for removal of formalin and nitric acid then preserved in 70% alcohol until paraffin embedding. Vertebrae were sectioned (10 µm thick) by using a rotary microtome (Model GE - 70). Mid-diaphyseal sections were stained with Harris haematoxylin and observed under compound microscope for enumeration of growth rings and photographed good sections. Growth rings in three calcified materials of each fish were counted independently by two readers without prior information of body weight and body length. Percent agreement between Otolith Ring Count (ORC) and Scale Ring Count (SRC), ORC and Vertebrae Ring Count (VRC) was calculated. Further, the relationship if any between BL and BW, otolith weight (OW) and BL; OW and BW; otolith diameter (OD) and BW; OD and BL,

and ORC and BL; ORC and BW was determined by calculating correlation coefficient '*r*' by Karl Pearson's method (Zar, 1996).

Results and Discussion: In the present study 134 fishes with mean body length of 13.55 cm and mean body weight of 138.5 gm were used. All three calcified materials showed growth rings each ring composed of faintly stained broader growth zone, and a darkly stained condensed line, lines of arrested growth (LAGS). Otolith was more or less circular in shape, thin and transparent in which clear and distinct growth rings were observed. One to four LAGs were noticed in fishes of different body sizes. Out of 134 fishes, 9 fishes with mean body length 10.75 ± 0.44 cm showed no LAGs in scales, otoliths and vertebrae; 31 fishes with mean body length 11.60 ± 1.25 cm exhibited one LAG each; 59 fishes with mean body length 12.24 ± 1.92 cm exhibited 2 LAGs; 27 fishes with mean body length 13.52 ± 2.49 cm showed 3 LAGs; and 8 fishes with mean body length 15.00 ± 2.81cm possessed 4 LAGs in scales, otoliths and vertebrae respectively (Table 1 and Figure 1).

In the present study ORC was compared with SRC and VRC for selection of most reliable calcified materials for accurate aging in this fish due to reason that otolith rings were more distinct, clear and easy to enumerate. Comparative study showed that number of growth rings were identical in otoliths and scales in 119 (88.81%) fishes out of 134 (Table 2). Remaining 15 fishes (11.19%) had one growth ring short in scales compared to otoliths in different age groups. This result indicates that increasing otolith age on one side and on other side decreasing percent agreement in scale age (Table 2). Vertebrae sections of *S. balookee* were circular with central marrow cavity which is surrounded by broad periosteal bone. Haemetoxylin stained sections showed clear and distinct growth rings in periosteal layers, *i.e.,* lighter stained growth zone and darkly stained LAGs. One to four LAGs were observed in vertebrae sections.

Comparative study showed that number of growth marks was identical in otolith and vertebrae sections in 133 (99.25%) fishes out of 134 (Table 2). Further, two fishes (1.49%) had an extra LAG in the periphery of periosteal layer in the four year age group individuals, but one (0.75%) fish had one LAG short compared to otolith in three year age group (Table 2). Percent agreement between otoliths and vertebrae age was 99.25% in this fish.

Randomly collected fishes for age determination indicated that 6.72% were in the first year, 23.13% in second year, 44.03% in third year, 20.15% in fourth year and 5.97% fishes in the fifth year of growth. There was a high degree of positive correlation between BL and BW (r = 0.94; Table 3). Otolith weight positively correlate with body length (r = 0.67) and body weight (r = 0.73) and OD with BW (r = 0.55) and BL (r = 0.55) respectively. Further, ORC is also showed moderate correlation with BL (r = 0.44) and BW (r = 0.47) (Table 3).

The presence of zero to five growth rings in scales, otoliths and vertebrae sections of freshwater fish *S. balookee* inhabiting the Sangli District, southern India showed similar to that reported in other tropical species namely, *Sillago japonica* (Sulistiono *et al*., 1999), Indian whiting, *Sillago indica* (David and Pancharatna, 2003), *Catla catla* (Ujjania, 2012), and *Notopterus notopterus* (Sudarshan and Kulkarni, 2013). The fact that most of the Indian freshwater fishes exhibit seasonal variation in body weight, gametogenic and breeding activity (Alam and Pathak, 2010; Ganie *et al.,* 2013), it suggests that formation of growth rings may be a cyclical phenomenon leading to the formation of LAGs. Therefore, the cyclic pattern in the growth rings in *S. balookee* may be due to the annual rainfall pattern which in turn may affect regular feeding activity by diverting the fishes towards reproductive activity. Akombo *et al.* (2015) have attributed that the formation of growth marks on hard structures in tropical fish as a result of reproductive activity, feeding intensity, lower salinity, increased turbidity and reduced

temperature during the rainy season in the water bodies. Also, factors like, environmental changes, food composition changes, competition with the food chain, changes in the physical and chemical properties of aquatic medium can influence on growth fluctuations in tropical and sub-tropical fishes (Abowei and Davies, 2009). The growth marks observed in this study may also be attributed to reduced feeding intensity, high turbidity and low oxygen during this period as this also coincided with the period of spawning. Furthermore, temperature variation of water may have impact in the deposition of calcium materials in otoliths and other calcified structures in fishes. The reduced water temperature might be responsible for poor deposition of calcium materials (formation of opaque zone) during rainy season (May – August), while increased water temperature might be influence to increasing the deposition of calcium materials (formation of transference zone) during remaining seasons (September - April) in tropical fishes. The reduction in water temperature is associated with reduced feeding activity of the fish, due to the poor plankton population during these months in the water bodies. Lombarte and Lieonart, (1993) suggested that otolith development occurs under dual regulation *i.e.* genetic conditions regulate the form of the otolith, while environmental conditions, mainly temperature in carbonate-saturated waters, regulate the quantity of material deposited during the formation of the otolith. Further confirmation study is necessary for seasonal variation in deposition of calcium materials in otolith or other hard calcified materials in this fish. Formation of annual growth rings in scales, otoliths and vertebrae of this fish might be controlled by food scarcity; feeding intensity, breeding activities, and reduced temperature in the river which coincide with the rainy season.

Further, experimental studies have confirmed that the formation of growth marks are annual in many fish species including, *Clarias gariepinus* (Wartenberg *et al*., 2011), *O. niloticus* (Abdel-Hadi *et al*., 2000) and *Puntius conchonius* (Bahuguna, 2013). Therefore, number of

LAGs present in these structures is directly depicted the age of individual fish. Presence of one to four LAGs suggests that this species may live for a maximum of five years. General trend in occurrence of growth rings in fishes indicates that large number of smaller sized fishes has lesser growth rings; whereas few larger sized fishes has more growth rings in their calcified materials (Table 1). The frequency distribution of these growth rings in the natural population further suggests that, always a large number of smaller sized fishes exhibited fewer growth rings and few large sized individuals possessed greater number of growth rings indicating the prevalence of a definite relationship between production and survival rate of this species.

Comparative study of otoliths vs scales and vertebrae showed that number of growth marks remained identical in otoliths and scales in 119 (88.81%) fishes, but 15 (11.19%) fishes showed one growth ring shorter in different age group fishes. Scale age underestimated compared to otolith age in this species due to the small sized transparent scale with un-cleared discontinuous growth rings. Kilambi and Prabhakaran (1989) have stated that enumeration of annuli on some scales in white bass (*Morone chrysops*) is very difficult to distinguish due to slow growths with length overlap of the constant age groups. Khan *et al.* (2015) found that the annual rings present in scales in *H. molitrix* are inferior as compared to other calcified structures. On the other hand, vertebrae sections provided age readings that were very close to (99.25%) those from otoliths. Growth rings are very clear and distinct in the vertebrae sections due to the wide periosteal layer. Similar results were reported in some fishes like, Polat *et al.* (2001) have reported that vertebrae are the most suitable and reliable aging structures in *Pleuronectes flesus luscus* compared to scales and otoliths. Bahugun (2013) has proved that the vertebrae are the most reliable structure for the age determination of *P. conchonius*. Khan *et al.* (2015) has reported that vertebrae were reliable age estimating structure compared to scales and otoliths in

Mastacembelus armatus and *Ompok pabda*. However, one (0.75%) fish showed one growth mark shorter in vertebrae compared to otolith in the three year age group individual, this might be due to technical problems related to histological processing. Further, two individual's (1.49%) possessed one growth ring extra in the four year age groups compared to ORC. Present investigation reveal that otoliths as well as vertebrae sections are most precise and reliable calcified materials for estimating age than scales in this species.

In order to study the longevity of fishes in natural populations, a random collection and large sample sizes (consisting of all the possible age groups) are advised to consider (Campana, 2001). The existing data on the longevity of tropical fishes in nature reveal that *S. indica, N. notopterus*, *Channa marulius, C. gariepinus* and *Oreochromis mossambicus* live for 5–6 years (David and Pancharatna, 2003; Sudarashan and Kulkarni, 2013; Lad, *et al.*, 2014) while other fishes such as *C. mrigala*, and *C. catla*, are reported to live longer, *i.e.* 8 - 9 years.

In conclusion presence of one to four annual rings in the three calcified structures in this fish population suggests that this fish live for a maximum of five years in nature. Formation of annual rings in the calcified materials of this fish appears to be influenced by scarcity of food; feeding intensity, breeding activities, and reduced temperature. Overall, results of the present study indicate that otoliths and vertebrae are most suitable aging materials compared to those of scales in *S. balookee*.

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Table 1: Otolith characteristics in relation with body weight, body length in the freshwater fish *S. balookee* (N = 134).

No. of LAGs	Age	Number of Fishes	%	Body Weight (gm)	Body Length (cm)	Otolith Weight (mg)	Otolith Diameter (mm)
0	1	9	6.72	35.67 ± 10.99	10.75 ± 0.44	0.76 ± 0.408	1.43 ± 0.25
1	2	31	23.13	47.37 ± 14.81	11.60 ± 1.25	1.13 ± 0.65	1.50 ± 0.24
2	3	59	44.03	61.18 ± 34.21	12.24 ± 1.92	1.37 ± 0.84	1.54 ± 0.32
3	4	27	20.15	88.40 ± 53.24	13.52 ± 2.49	1.78 ± 1.21	1.64 ± 0.34
4	5	8	5.97	115.47± 58.46	15.0 ± 2.81	2.37 ± 1.34	1.86 ± 0.23

Note: Values are means ± SD.

Table 2: Agreement and disagreement of scales and vertebrae ages with otolith age in *S. balookee* (N = 134).

Otolith Age	Number of Fishes	Percent Agreement	Remarks
Scale Ages			
I	09	100	---
II	31	98.51	1.49(2) UE
III	59	96.27	3.73(5) UE
IV	27	95.52	4.48(6) UE
V	08	98.51	1.49(2) UE
Vertebrae Ages			
I	09	100	---
II	31	100	---
III	59	99.26	0.74(1) UE
IV	27	100	---
V	08	98.51	1.49(2) OE

Note: Number in parenthesis indicates that number of fishes; UE = Under Estimation and OE = Over Estimation

Table 3: Correlation between body length and body weight, otolith diameter with body length, weight, and otolith ring count with body length and weight in *S. balookee* (N = 134).

Sr. No.	Correlation between	'*r*' value	Remarks
1	Body Length and Body Weigh	0.94	Highly correlation
2	Otolith Weight with Body Length	0.67	Good correlation
3	Otolith Weight with Body Weight	0.73	Highly correlation
4	Otolith Diameter with Body Weight	0.55	Good correlation
5	Otolith Diameter with Body Length	0.55	Linear correlation
6	Otolith Ring Count with Body Length	0.44	Moderate correlation
7	Otolith Ring Count with Body Weight	0.47	Moderate correlation

***** Correlation coefficient '*r*' was calculated using Karl Pear

Figure 1: Relationship between Body Length (BL) and Body Weight (BW) in *S. balookee*

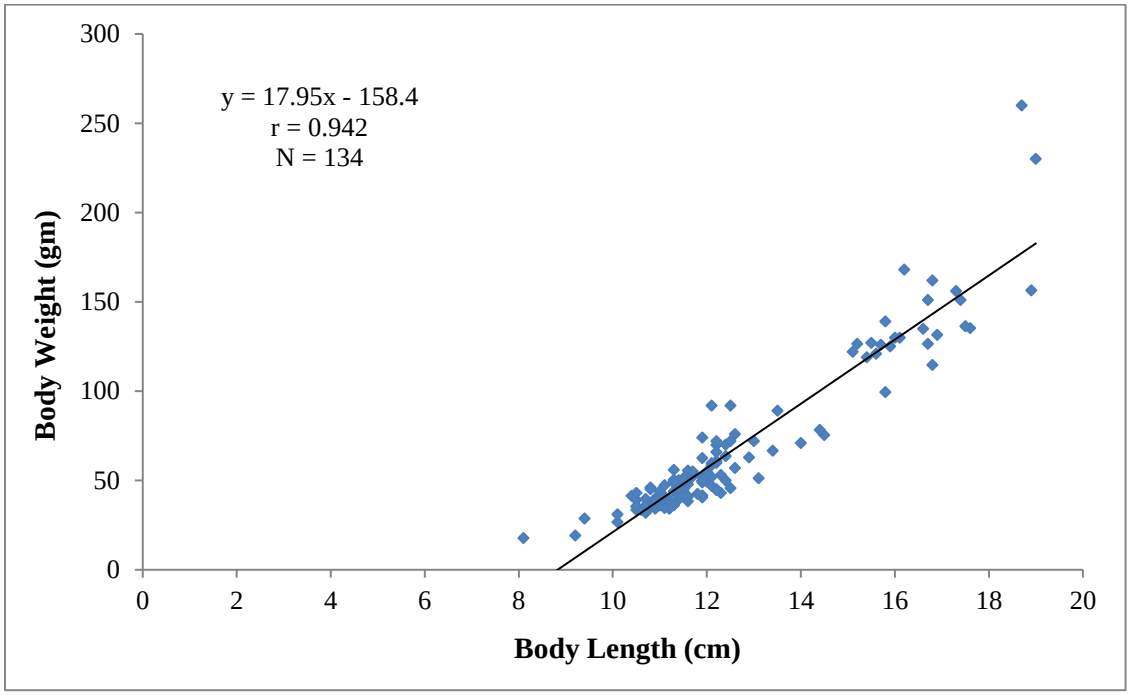


Figure 2: Relationship between Body Length and Otolith Weight in *S. balookee*

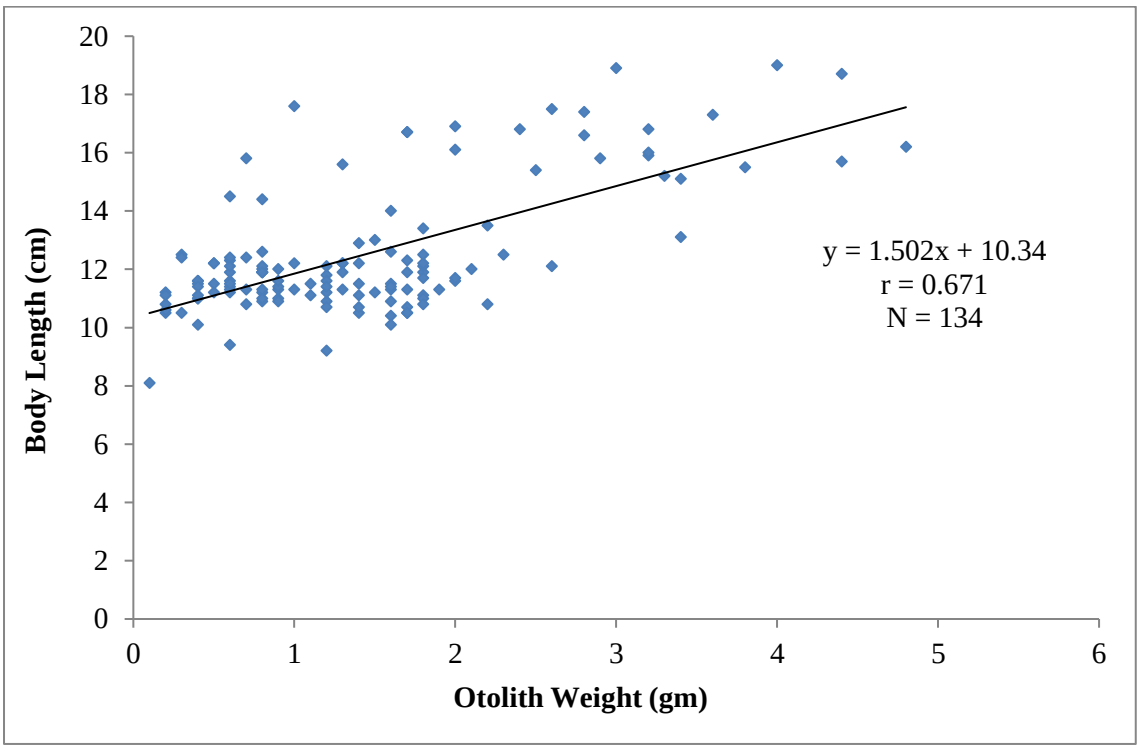


Figure 3: Relationship between Body Weight (BW) and Otolith Weight (OW) in *S. balookee*

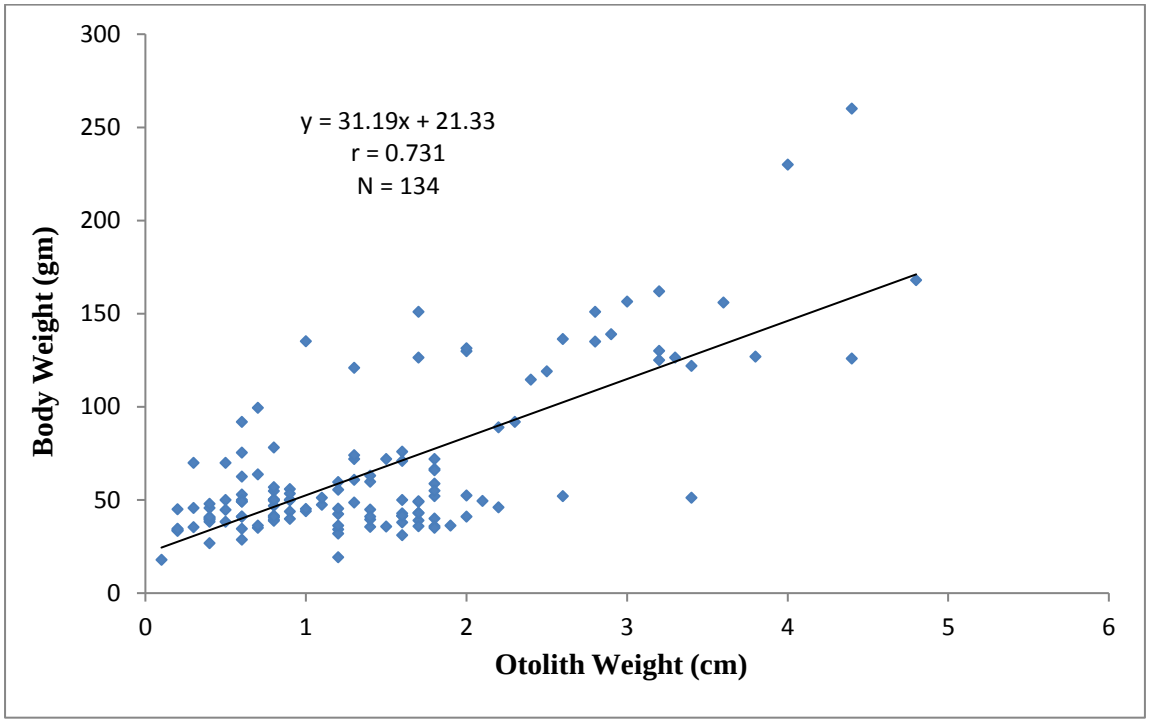


Figure 4: Relationship between Otolith Diameter (OD) and Body Length (BL) in *S. balookee*

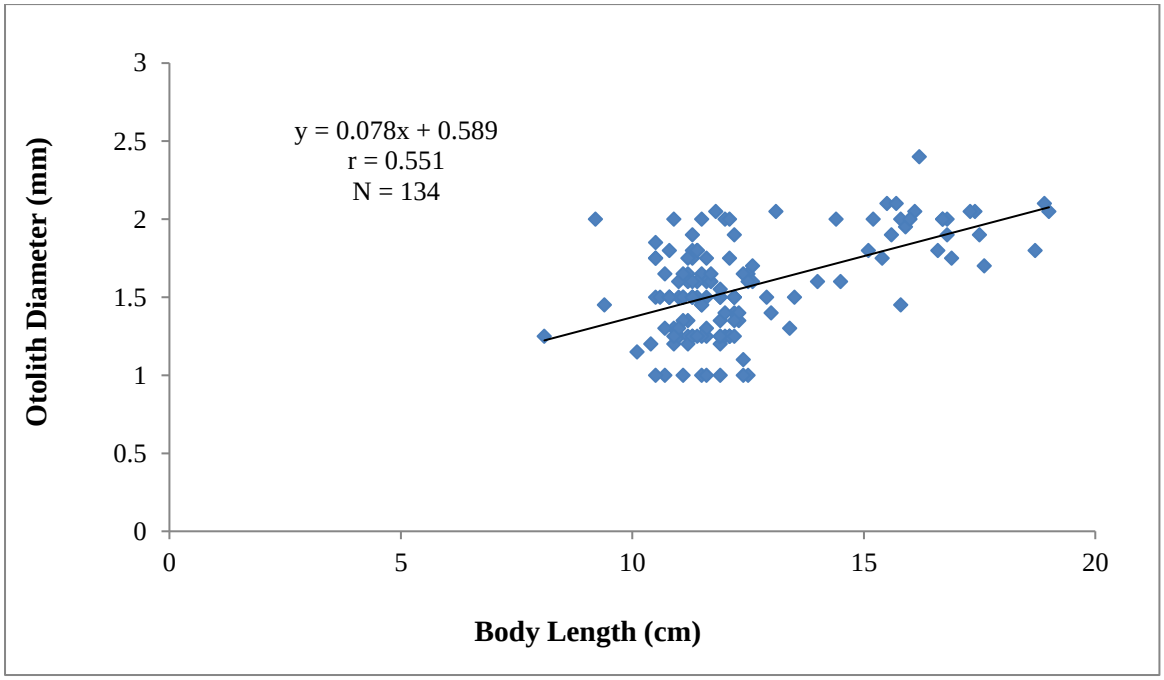


Figure 5: Relationship between Otolith Diameter (OD) and Body weight (BW) in *S. balookee*

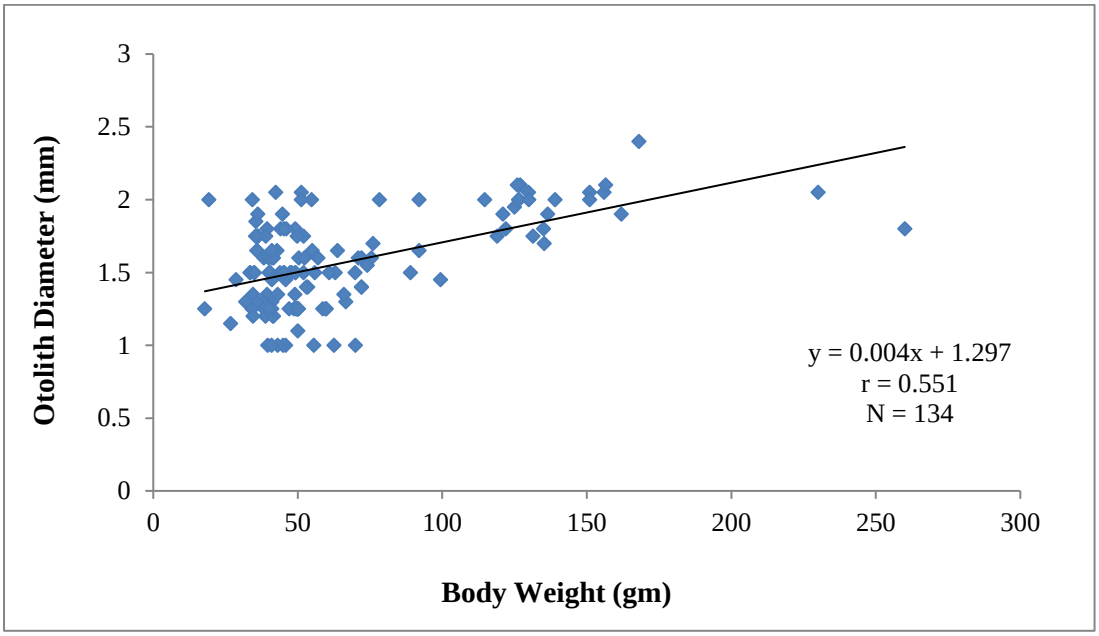


Figure 6: Relationship between Otolith Ring Count (ORC) and Body Length (BL) in *S. balookee*

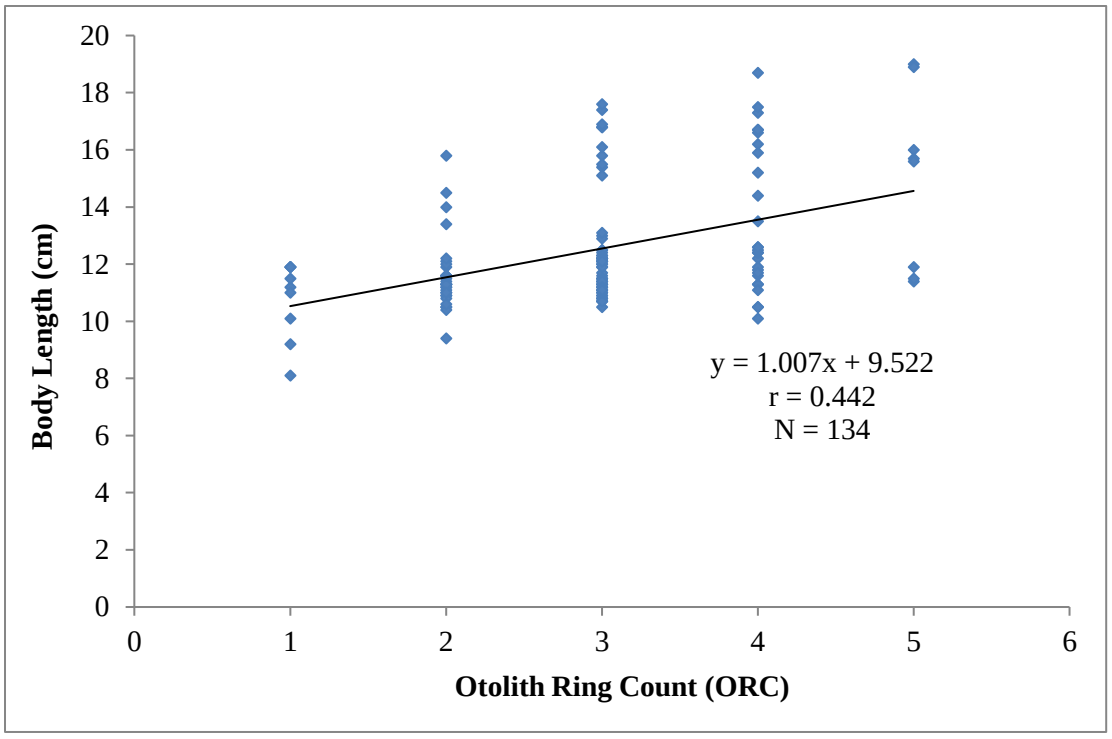
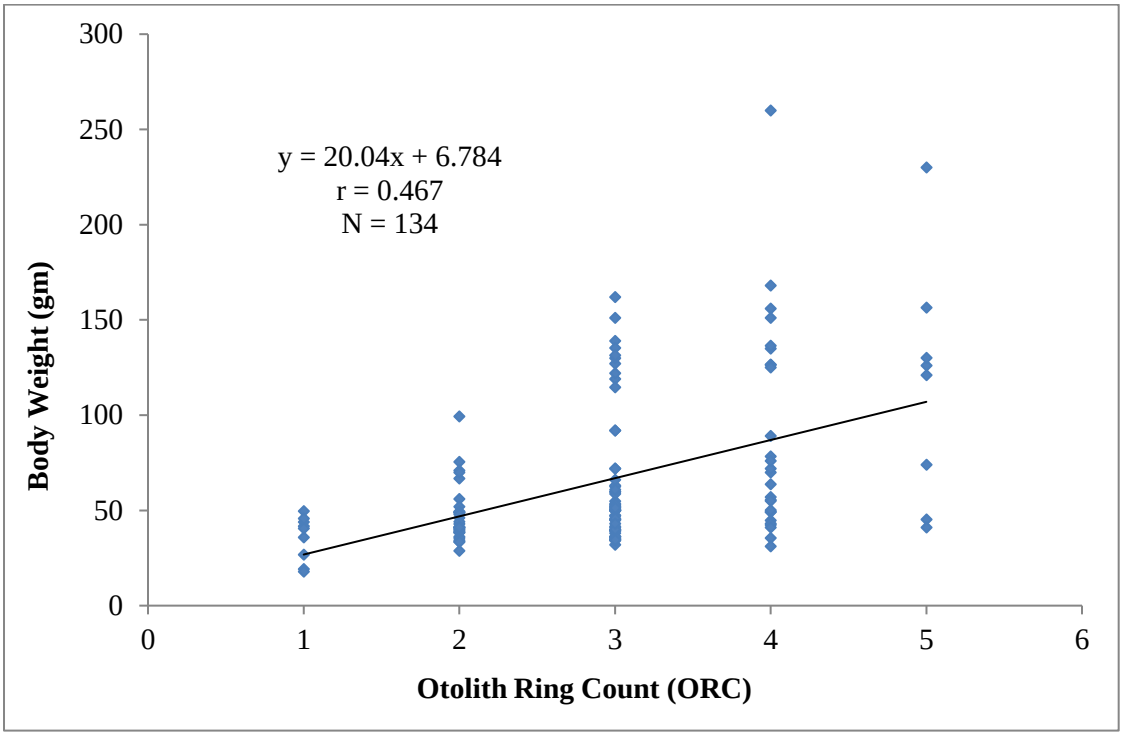


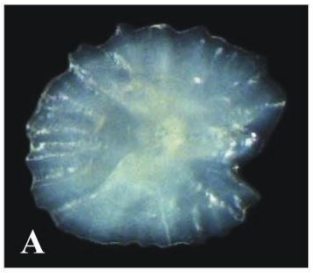
Figure 7: Relationship between Otolith Ring Count (ORC) and Body Weight (BW) in *S. balookee*



Explanations to Plate

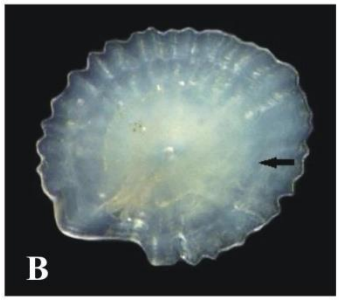
Plate 1A-O: Otoliths, scales and vertebrae cross-sections of freshwater fish *S. balookee* 1A, F, K- No LAG in otolith, scale and vertebrae of same fish; **B, G, L** with 1LAG; **C, H, M** with 2 LAGs; **D, I, N** with 3 LAGs and **E, J, O** with four LAGs in otolith, scale and vertebrae section. Scale line = 100 µm; LAGs - Lines of arrested growth.

PLATE - 1


A

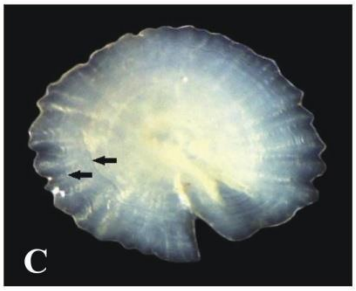
F

K


B

G

L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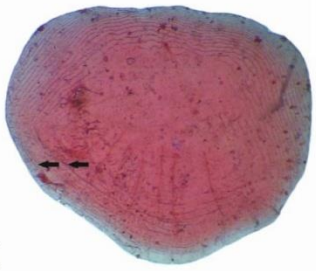
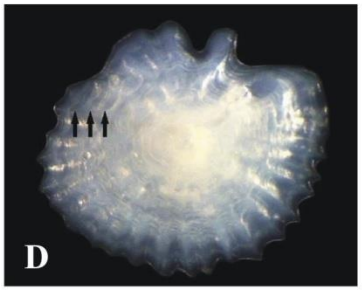

H

M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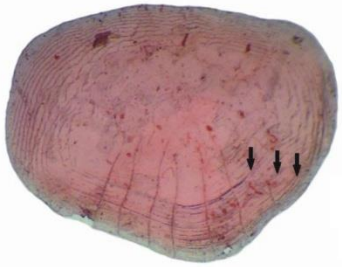
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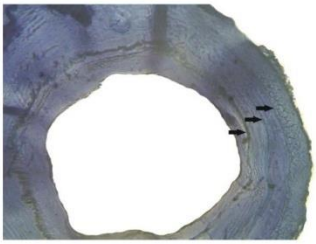
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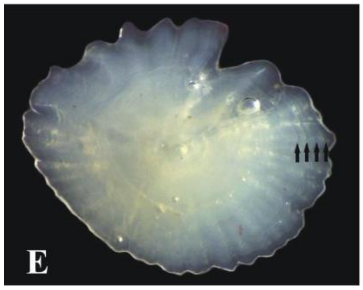
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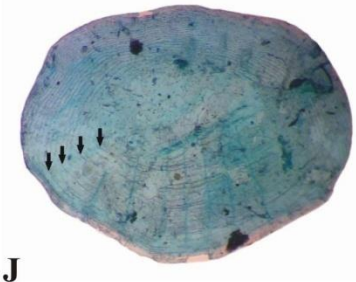
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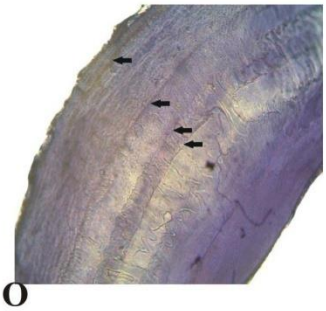
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Topic - III

Determination of Age and Longevity of Road Mortal Indian Common Toad *Duttaphrynus melanostictus* by Skeletochronology

Abstract: Age and longevity of road mortal Indian common toad, *Duttaphrynus melanostictus* was determined by skeletochronology. Ninety three road mortal toads were used for longevity study from the Sangli District (17^0 09' N & 74^0 45' E) Western Maharashtra, Southern India. In the laboratory, body mass and size of each toad were recorded, the 4^{th} toe of hind limbs were clipped and processed for histology. Sections of 8 µm thickness were stained with Harris haematoxylin. Mid-diaphyseal sections of phalanges exhibited growth rings, each consisting of a broader growth zone, and a chromophilic line of arrested growth (LAG). Among the toads studied, 25.86% showed no LAGs, 30.10% one LAG each, 23.65% two LAGs, 13.98% three LAGs, 5.38% four LAGs and 1.07% of toads exhibited nine LAGs in their phalangeal histology. Back calculation indicated that the first LAG was partially eroded in 9 (9.67%) individuals due to resorption. This toad can live more than ten years in natural population.

***Key words**:* Amphibia: Anura: *Duttaphrynus melanostictus*, age, longevity, skeletochronology

Introduction: The Indian common toad *Duttaphrynus melanostictus* is a widely distributed species in Taiwan, southern China, Hainan Island, southward through Southeast Asia to Indonesia, and also in westward to India and Sri Lanka (Shieh, 1993). It has been recorded from up to 1800 m ASL. The toad is widely used as a model for research in physiology and reproduction because of its easy availability. A lot of work has been carried out in the field of diversity (Khan, 2000), reproduction (Kanmadi and Saidapur, 1982; Saidapur and Girish, 2001; Ngo and Ngo, 2013), and road mortality studies (Baskaran and Boominathan, 2010) in this species. However, studies on aging of Indian common toad are poorly understood. In the present day high rate of herpetofaunal mortality has been occurring on the roads due to heavy vehicular traffic in which amphibians were the most affected taxa (Das et al., 2007). Among the amphibians especially Indian common toad *D. melanostictus* was recorded highest rate of mortality; it may be due to slow to react to vehicles and this along with the drivers' ignorance (Basakaran and Boominathan, 2010). However, Bhupathy et al. (2011) have recorded more than 42 road mortally *D. melenostictus* out of 110 vertebrate species during dry and wet seasons along the National Highway 220 which cuts the Western Ghats of India. (Basakaran and Boominathan, 2010) have also recorded high percent of amphibian mortality (53%) followed by reptiles (22%), mammals (18%); including a leopard (*Panthera pardus*) and birds (7%) in the tropical forests of Mudumalai Tiger Reserve, southern India by vehicular traffic. We have also observed higher mortality in amphibian's especially *D. melanostictus* in Sangli and Karad road and other roads of Sangli district. The rate of mortality was very high at an early morning time in the monsoon season (June to September). For the first time we are using road mortal Indian common toad *D. melanostictus* for determining the age and longevity by phalangeal skeletochronology.

Materials and Methods: We were collected 93 road mortal Indian common toad *D. melanostictus* (body mass: 5 - 600 g; body size [snout-vent-length, SVL]: 3.5 - 15 cm) from the Sangli to Karad road (SH No. 75) at an early morning during rainy seasons (June 2013 to September 2015). There were different range of damages in the road mortal toads among them we were picked up least damaged specimens and kept in plastic bags and brought to the laboratory where the body mass (to the nearest gm) and snout-vent length (SVL, measured to the nearest cm using a thread) of each toad were recorded. The 4^{th} toe of right or left hind limb (depending upon availability) of each toad were clipped and fixed in 10% formalin and numbered serially. Clipped toes were cleaned and demineralized in 5% nitric acid and processed for histology. Paraffin sections of 8 µm thickness were cut on a rotary microtome and stained with Harris haematoxylin. Mid-diaphyseal sections of phalanx were chosen for observation under a compound microscope and presence of number of growth rings (LAGs) was enumerated. The relationship between the body mass vs SVL and number of growth marks versus body mass and body size were assessed by drawing scatter plots and calculating correlation coefficients (r) by Karl Pearson's method (Steel and Torrie, 1980).

Results: Mid-diaphyseal cross-sections of phalanges of *D. melanostictus* showed growth rings, each ring composed of a faintly stained broader growth zone, and a darkly stained condensed chromophilic line, the LAG (Plate 1A-D). All toads possessed exclusively as single LAG in the phalangeal histology. Among the toads studied, 24 toads (25.86%, SVL: 4.11 ± 2.08, Plate 1A) showed no LAGs, 28 toads (30.10%; SVL: 7.36 ± 1.78, Plate 1B) possessed one LAG each, 22 toads (23.65%; SVL: 7.12 ± 1.58, Plate 1C) exhibited two LAGs, 13 toads (13.98%; SVL: 9.7 ± 1.64) showed three LAGs, five toads (5.38%; SVL: 9.48 ± 2.66) possessed four LAGs and one toad (1.07%; SVL: 15.2) exhibited nine LAGs in their phalangeal histology (Fig. 1; Table 1;

Plate 1D). The distance between two LAGs was much variable in periosteal bone the thickness of the growth layers progressively declined from the inner endosteal bone to outer periosteal bone layer, representing a decrease in bone growth in older individual (Plate 1D). Histology of the distal phalanx of the smallest (body mass: 4 g, SVL: 3.5 cm) toad of our study showed a large marrow cavity in the center, circumference by a thin periosteal bone (Plate 1A). When 'back calculation' was made in order to estimate the loss of LAGs if any due to endosteal resorption, it was found that the first LAG was partially eroded in 9 (9.67%) individuals it was also confirmed by phalangeal histology (Plate 1C & D). There was a high degree of positive correlation between the body mass and body size (r = 0.77; Fig. 2) and number of growth marks and body mass (r = 0.84, Fig. 2) and body size (r = 0.68, Fig. 3) respectively.

Discussion: Phalangeal skeletochronology is successfully applied for studying rare and endangered species and also fossil specimens of amphibians and reptiles (Peabody, 1961; Castanet and Smirina, 1990; Smirina, 1994; Guarino and Erismis, 2008). The validity and reliability of skeletochronology in the determination of age of amphibians and reptiles have been thoroughly reviewed (Halliday and Verrell, 1988; Castanet and Smirina, 1990; Smirina, 1994; Sinsch, 2015). However, a majority of aging studies on amphibians concentrate from temperate zones, where drastic fluctuations in the ambient temperature become a limiting factor for feeding activity and enforce the formation of seasonal bone growth for instance, (Hemelaar, 1981, 1988; Smirina, 1983, 1994; Francillon et al., 1984; Castanet and Smirina, 1990; Cherry and Vieillot, 1992; Wake and Castanet, 1995; Tejedo et al., 1997; Sinsch, 2015). Comparative studies on tropical amphibians are limited (Halliday and Verrell, 1988; Smirina, 1994; Sinsch, 2015). However, few skeletochronological studies are available on tropical anurans (Kulkarni and Pancharatna, 1996; Guarino et al., 1998; Pancharatna et al., 2000; Kumbar and Pancharatna,

2001a, 2002; Pancharatna, 2002; Lai et al., 2005; Guarino and Erismis, 2008; Guarino and Erismis, 2014; Ashkavandi et al., 2012). Preliminary skeletochronological observations on Indian common toad *D. melanostictus* inhabiting southern India reveal the presence of growth marks consisting of broader growth zones and chromophilic LAGs in the cross-sections of phalanges and limb bones (Kumbar and Pancharatna, 2001a). This toad shows a clear-cut seasonality in body mass, fat body mass and gamatogenetic activities (Kanmadi and Saidapur, 1982; Kumbar and Pancharatna, 2001a) suggesting indirectly that bone growth is a cyclical phenomenon. Moreover, formation of growth mark is annual has been experimentally confirmed in this toad (Kumbar and Pancharatna, 2004).

It is known that structural remodeling in bones and the rate of endosteal resorption severely influence skeletochronological interpretations in determining the age of amphibians (Halliday and Verrell, 1988; Castanet and Smirina, 1990; Smirina, 1994; Sinsch, 2015). Previous comparative studies have been confirmed that phalangeal bone is a most reliable material for age estimation in this toad because number of growth marks in long bones (femur, tibio-fibula, humérus, radioulna) and phalanx was identical within specimens (Kumbar, 2002; Nayak et al., 2007). In the present study the rate of endosteal resorption is assessed based on the comparison between the phalangeal sections at the same magnification from different individuals; we estimated that the first (innermost) periosteal LAG is partially eroded in nine (9.67%) individuals. There is no loss of complete LAG due to resorption therefore; enumeration of periosteal LAGs is directly depicted the age of individual toad. As a consequence out of 93 toads, 26% of toads were in the first year, 30% in second year, 24% in third year, 14% in fourth year, 5% toads in the fifth year and 1% in tenth year of growth. Therefore, in the natural population this species may live for a maximum of 10 years. Earlier our preliminary

skeletochronological record of adult toad *D. melanostictus* inhabiting Dharwad southern India has been described that the oldest toad to be five years in contrast to the present investigation where as the oldest toad is found to be ten years. In our previous study sample size was very less (N = 50), and body size was also (SVL: 3.0 – 11.0 cm) smaller compared to the present sample size (N = 93) and body sizes (SVL: 3.5 – 15.0 cm). A Dharwad (15° 17' N, 75° 03' E) and Sangli (16^0 75’ N & 73^0 70’ E) districts are located in the Southern region of India and there are no drastic changes in geographical and climatic conditions. Beside, toad *D. melanostictus* [SVL: 1.5-10.4 cm; N = 68] inhabiting in Bhubaneswar (20° 18' N, 85° 50' E) Eastern India live for maximum 12 years (Nayak et al., 2007). This difference in longevity in anurans may be related to the geographical position of the respective regions because in the south, the annual variation in mean temperature normally does not exceed 10°C whereas the variation in temperature is around 25°C in the eastern part of India. This pattern may be suggests that lower temperature and smaller food availability from high altitude may increase longevity and decrease the body size. Similarly, body size and longevity changes with altitude in most anuran individuals from high-altitude populations having longer longevity, slower growth rate and larger body size than low-altitude populations due to lower temperature and smaller food availability in high altitude (Miaud et al. 1999; Lu et al. 2006; Matthews and Miaud, 2007; Liao and Lu, 2010a). However, comprehensive study is needed to confirm whether geographical variations, environmental condition, availability of food and genetic factor controlling body growth and longevity in tropical anurans.

There was a positive correlation between the body weight and body size (r = 0.77) and number of growth marks and body weight (r = 0.84) and body length (r = 0.68) respectively. This result suggests that body weight and size may be a reliable criterion for aging in *D.*

melanostictus unlike in other southern Indian species such as *E. cyanophlyctis, M. ornata, H. tigerinus, Polypedates maculatus* in which body size correlated positively with the number of LAGs (Kulkarni and Pancharatna, 1996; Kumbar and Pancharatna, 2001a; Kumbar and Pancharatna, 2002; Pancharatna and Kumbar, 2005). Similarly, Indian green frog *E. hexadactylus, P. maculatus* and *D. melanostictus* inhabiting eastern region of India showed high degree of positive correlation between body mass and body size (SVL) as well as body mass and body size with number of LAGs (Nayak et al., 2007; Nayak et al., 2008; Mahapatra et al., 2008). By contrast, few species *Rana sylvatica* and *L. limnocharis* (Boie, 1835) showed very poor correlation between body size and age (Leclair et al., 2000; Pancharatna and Deshpande, 2003). Moreover, there is often a wide size overlap among age classes even if body size and age are positively correlated. In conclusion, phalangeal skeletochronology can be successfully applied for age determination in road mortal Indian common toad *D. melanostictus.* Further, there is need to confirm whether geographical variations, environmental condition, availability of food and genetic factor controlling body growth and longevity in this toad.

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Table 1: Body weight (g), body size (SVL, cm), number of Lines of arrested growth marks (LAGs) and age in a sample of 93 specimens of *D. melanostictus*

No. of LAGs	Age	Number of Toad	Body Weight (gm)	Body Length (cm)	Percentage (%)
0	1	24	8.14 ± 7.40	4.10 ± 2.08	25.806
1	2	28	25.71 ± 11.88	7.36 ± 1.78	30.107
2	3	22	31.17 ± 13.79	7.12 ± 1.58	23.655
3	4	13	60.61 ± 16.30	9.7 ± 1.64	13.978
4	5	5	66.02 ± 24.74	9.48 ± 2.66	5.376
8	9	1	150 ± 0	15.2 ± 0	1.075

Figure 1: The graph shows the distribution of the toads (*Duttapherenous melanostictus;* N = 93) according to different age groups.

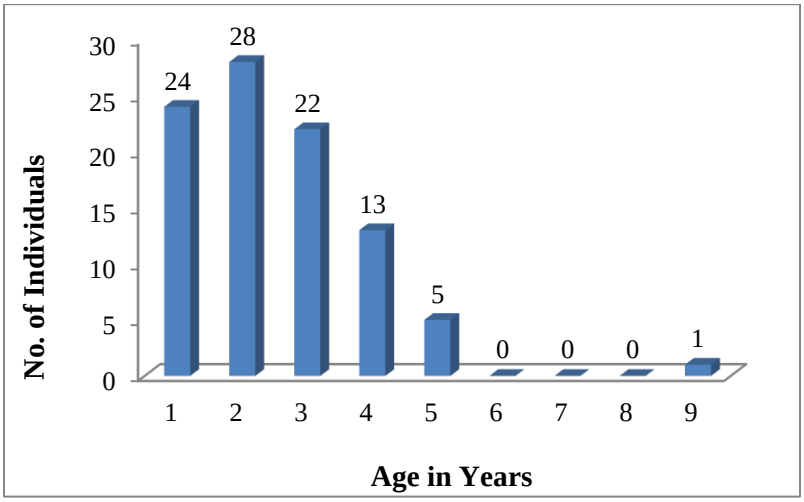


Figure 2: Correlation (r = 0.77) between body size (SVL, cm) and Body weight (gm) in *D. melanostictus*.

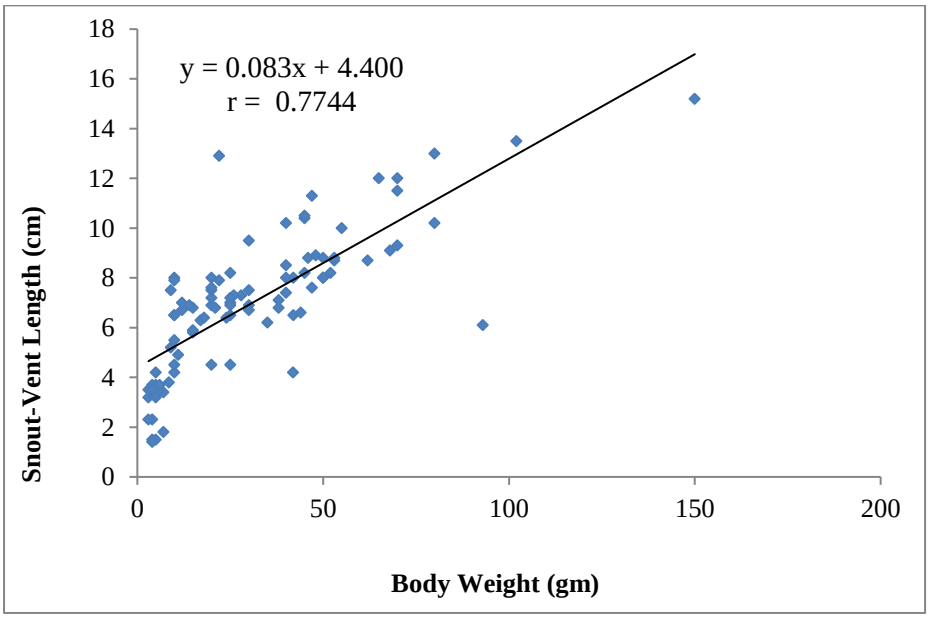


Figure 3: Correlation (r = 0.83) between body weight (gm) and number of growth marks in *D. melanostictus*.

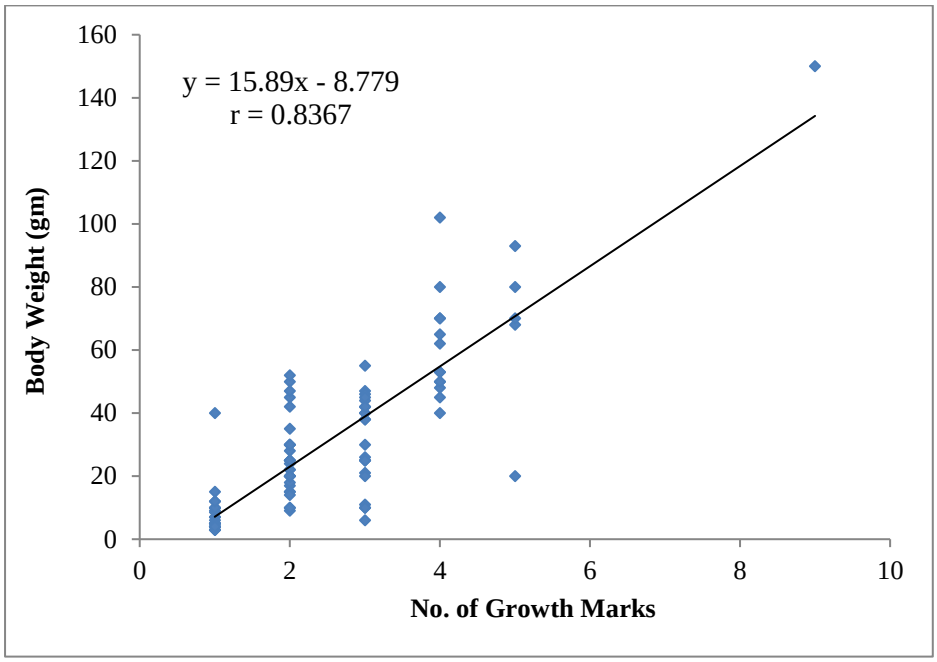
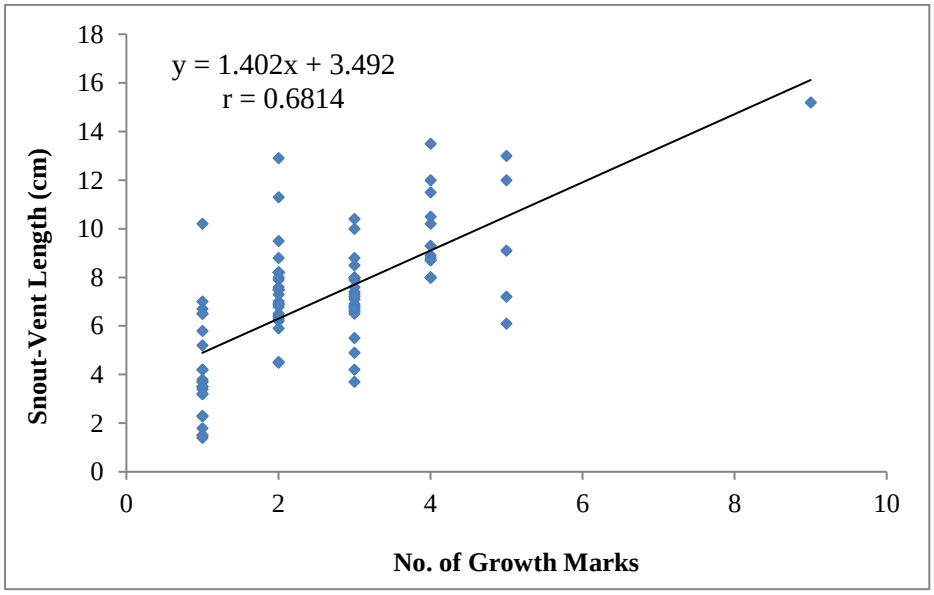


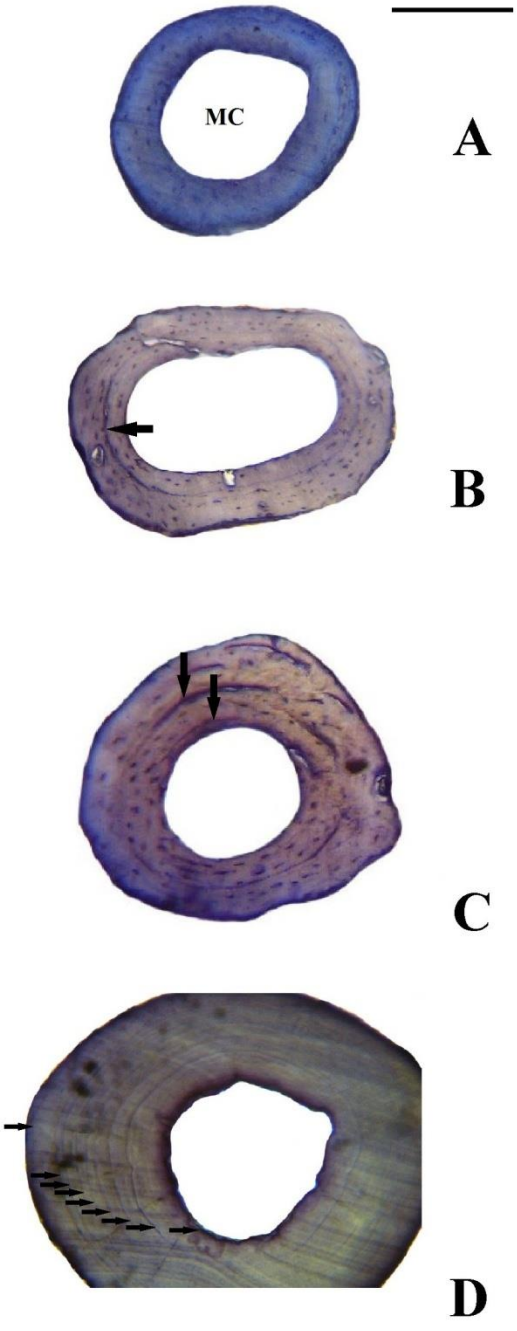
Figure 4: Correlation (r = 0.68) between body size (SVL, cm) and number of growth marks in *D. melanostictus*.



Explanations to Plate

Plate 1A-D: Cross-sections of distal phalanges of *D. melanostictus*: 1A- Phalangeal histology showing no LAG (smallest sized toads in the sample), 1B - with one LAG, 1C -two LAGs, 1D - with nine LAGs; Scale line = 100 µm; LAGs - Lines of arrested growth.

PLATE - 1



Topic - IV

Age and Longevity of Indian Garden Lizard *Calotes versicolor* by Skeletochronology

Abstract: In this study, age structure and longevity of 40 individuals (22 males and 18 females) of Indian garden lizard *Calotes versicolor* was determined by skeletochronology. *C. versicolor* is a medium-sized, arboreal lizard with oval head and laterally compressed body. They are commonly found among the undergrowth in open habitats including highly urban areas. Mainly they feed insects and small vertebrates, including rodents and other lizards. Occasionally this lizard also consumes vegetable matter. Its average snout vent length (SVL) was 9.49 ± 1.74 cm and 8.35 ± 1.07 cm, whereas the median age was 2.77 ± 1.31 (range = 2 - 5) for males and 2.32 years (SD = 1.04, range = 2 - 4) for females, respectively. No statistically significant differences were noticed in body mass and SVL between the sexes. However, there was a significant positive correlation between body mass and SVL (r = 0.86). Based on this study, the maximum longevity of this lizard is from 5 years for females to 6 years for males in a natural population.

Key words: *Calotes versicolor*, tropics, age structure, skeletochronology

Introduction: India, being a mega-diverse country, harbors more than 518 species of reptiles (Aengals et al., 2007). Among these reptiles, the Indian garden lizard *C. versicolor* (Daudin 1802) is a largely widespread tropical lizard and found in the Indian subcontinent (Auffenberg and Rehman, 1993; Venugopal, 2010). However, it ranges from South-eastern Iran, Afghanistan, Bangladesh, Pakistan, Nepal, Bhutan, Sri Lanka, Myanmar, Thailand, Western Malaysia, Maldives, Vietnam, Cambodia, South China, Indonesia, Mauritius. Recent field survey confirms its distribution to Oman, Singapore and United states (Radder, 2006). The body color of *C. versicolor* is light brown and grayish on dorsal side with transverse spots on back and sides (Tikader and Sharma, 1992). These lizards have adapted special behavior in field; they run with a considerable speed and on the approach of danger dash away with tail tip erect, until they find refuge in some bushes or crevices in the ground. When running quickly they often adopt bi-pedal mode of locomotion. Genetic investigations of the genus *Calotes,* in Southeast Asia are still very limited. However, studies by Zug et al., 2006 confirmed that *C. versicolor* is a complex of multiple species which necessitates fixing type locality and specimen for the species in order to revolve the systematic of the species complex. A population genetics study performed on *C. versicolor* from China and Vietnam provided evidence for high intra-populational genetic diversity and high genetic differentiation between populations (Huang et al., 2013). Since *C. versicolor* is so widespread due to its ability to remain relatively unaffected by human activities and urban development, it has had a significant effect on other species. For example, *C. versicolor* was so successful after being introduced on Singpore Island that it has nearly out competent the native agamid lizard, *Bronchocela cristatella* (Ji et al., 2002b). *C. versicolor* has also had a significant negative effect on the other native and introduced reptile species in southern Florida (Enge and Krysko, 2004). This lizard is purely seasonal breeder. Both the sexes

develop red color on antero-dorsal region during breeding season, a sign of onset of sexual maturity (Pandav et al., 2007). The males exhibited red colored hues on the head and gular area. The lizards began exhibiting courtship behavior within a year. It has longer breeding season coinciding with the South West monsoon (May to October) and lays flexible shelled eggs by digging the nest hole up to 5-9 cm deep (Pandav et al., 2010). The adult female lizard is a multi-clutched lizard with clutch size varying from 7–33 eggs (Shanbhag et al., 2000). A lot of work has been carried out in the various fields on this Lizard. However, there are no skeletochronological age structure studies on the tropical garden lizard *C. versicolor.* The present study aims to determine age, longevity and relationship between body mass and size between the sexes in *C. versicolor*.

Materials and methods: Study Area: Indian garden lizard *C. versicolor* were collected from the forest area of Yasvantrao Chavan Sagreshwar Wildlife Sanctuary, Devrastre (17^0 09' N and 74^0 45' E), Sangli District, India in the month of April. Study area has rich vegetation along with low and high elevations. The type of vegetation is dry deciduous and mixed type forest. It receives an annual rainfall about 300–500 mm from June–October and frequently undergoes drought condition. The maximum recorded temperature was up to 41°C during summer and a minimum temperature of 10°C in winter (Kumbar and Patil, 2011). Population of this species is very prosperous in the study area due to availability of food sources around the year. There is no clear sexual size dimorphism in a population. Collected lizards were brought to the laboratory where the sex of each individual was assessed from their breeding behavior and cloacal morphology. Males have an elongated cloaca whilst that for females is swollen and round. Base of the tail is swollen in males compared to that of females. Simultaneously the body size (snout-vent-length, SVL) was measured to the nearest 0.1 cm with a digital caliper and body mass was

measured to the nearest 0.1 g with a single pan balance, the 4th toes of right limb was chopped off under light ether anesthesia (Comas et al., 2016) and fixed in 10% formalin for histological process. The lizards were kept under observation until the recovery and then allowed to release at the site of collection. The formaldehyde fixed toes were washed in running water for 24 hrs, decalcified in 5% nitric acid and then washed in running water for 24 hrs. The resulting phalangeal bones were then cross-sectioned (10 μ thick) on a rotary microtome (Model GE-70), then sections stained with Harris hematoxylin for 10-15 min. Sections from the mid-diaphysis were selected and mounted in glycerin after rinsing with tap water and observed with light microscopy (OLYMPUS CX-41) and enumerated the number of LAGs. For each individual, at least 5 sections were scored to check for consistent reproducibility and reduce the risk of errors due to localized breakage of LAGs by endosteal resorption. Photomicrographs of representative sections were taken with a digital camera (ABBOT DEC – 2000). Students-*t* test was carried to determine the significant difference between the sexes in body mass, body size (SVL) and LAGs. Furthermore, relationships between body mass vs body size and body size vs LAGs were calculated by Karl Pearson's correlation coefficient 'r'. Statistical analyses were performed by SPSS (Version 10.0).

Results: Among 40 individuals with sex ratio of 22 males and 18 females, the mean SVL was 9.49 ± 1.74 cm and 8.35 ± 1.07 cm for males and females, respectively (Table 1). The median age was calculated as 2.77 years (SD = 1.31, range = 2 - 5) for males and 2.32 years (SD = 1.04, range = 2 - 4) for females (Table 1). The hematoxylene stained phalangeal cross sections consisted of, a light wide zone representing a season of rapid growth; and a thin, dark zone representing a season of slow growth, making up a single year's growth. Zero to five LAGs was observed in the cross sections of phalanges of different sized individuals (Fig. 1 A-D). The

maximum age found was 5 years for females and 6 years for males (Table 2; Fig. 2). Life expectancy of this species was compared to the other tropical lizard species which were previously assessed by skeletochronology (Table 3). The LAGs were closer together near the margin of the bone opposite from the bone marrow cavity and so the distance between inner was much larger than that for outer periosteal layer in both sexes (Fig. 1D). In 5 individuals (12.5%) cross sections, the first LAG was partially resorbed by the endosteal bone but did not disappear completely (Fig. 1C). There was a positive correlation between body mass and SVL in male (r = 0.61) and female (r = 0.86). Males were larger than females but there was no significant difference between body mass (t = 2.3761, df = 38, P < 0.0226) and SVL (t = 2.5262, df = 38, P < 0.0158) between the sexes (Table 1).

Discussion: Determining the age of individual animal is extremely important for life history and population dynamics studies in amphibians and reptiles (Smirina, 1994; Smirina and Castanet, 1990). Various methods like; body size analysis, testis lobation, mark-release-recapture and skeletochronology are employed for estimating age in reptiles. Mark-release-recapture is most useful method among the above (Durham and Benett, 1963). However, this method is time consuming and requires an important amount of field hours to reach the results (Comas et al., 2016). Alternatively, skeletochronolgy is widely used method for age estimation in amphibians and reptiles (Smirina, 1974, Castanet and Smirina, 1990; Castanet et al., 1993; Castanet, 1994; Snover and Rhodin, 2008; Guarino et al., 2010). Moreover, skeletochronology is proved to be reliable and accurate method for assessment of age in temperate as well as tropical reptilian species (Smirina and Ananjeva, 2001; Smirina and Ananjeva, 2007; Guarino et al., 2004; Guarino et al., 2010). In India, skeletochronological studies are more concentrated on anuran species (Kulkarni and Pancharatna, 1996; Kumbar and Pancharatna, 2001a; Kumbar and

Pancharatna, 2001b; Pancharatna and Deshpande, 2003; Nayak et al., 2007; Nayak et al., 2008). However, this techniques have been successfully applied to few Indian reptilian species, for instances, *Psammophilus dorsalis* (Mahapatro et al., 1989), male *C. versicolor* (Patnaik and Behera, 1981), Fan-throated lizard, *Sitana ponticeriana* (Rath and Pal, 2007; Pal et al., 2009), *Hemidactylus brooki* (Pancharatna and Kumbar, 2005) and Indian skink, *Mabuya carinata* (Kumbar, 2010).

The results of the present study demonstrate that growth marks comparable to those reported in previous reptilian species inhabiting north east region of India are detectable in the cross sections of phalanges of south Indian garden lizard *C. versicolor*. The fact that many of the southern Indian reptilian species exhibit marked seasonality in the reproductive activity and abdominal fat body mass (Sarkar and Shivanandappa, 1989; Shanbhag and Prasad, 1992; Pandav et al., 2010), suggests that bone growth is a cyclical phenomenon in these animals. However, both body and fat-body mass of the lizard were very lower in late breeding phase (May – August), which coincides with the onset of monsoon rains; from September onwards there is an increase in body and fat-body mass, and they attain their maximal values between December and April (Shanbhag and Prasad, 1992; Shanbhag, 2003). Therefore, in this lizard, the LAG(s) may be laid down between May - August when the body growth and food reserves almost ceased coinciding with the wet season of the year. From September onwards when body and fat-body masses start to restore, the next osteogenic cycle may begin. Differences in life expectancy of Indian *C. versicolor* between the sexes have been found in two populations. The estimated longevity was 6 years in males and 5 years in females in the present study. These results reveal that male lizard population of South India is 2 years older than that of the north east region male population of *C. versicolor* (Patnaik and Behera, 1981). Similarly, Guarino et al. (2010) found

that age ranged from 2 to 4 years in males and from 2 to 3 years in females for a population of *Lacerta agilis* from Italy. However, in *L. agilis* living in Russia the maximum longevity observed was 6–7 years for males and 5–6 years for females, depending upon altitudes (Roitberg and Smirina, 2006). Longevities found for *C. versicolor* are similar to the other southern sympatric species (*H. brooki* and *M. carinata*), assessed by means of skeletochronology (Pancharatna and Kumbar, 2005; Kumbar, 2010). The longevity determined for *P. dorsalis* and Fan-throated lizard, *S. ponticeriana* from high altitudes of north east India ranges from 4 to 5 years (Mahapatro et al., 1989; Rath and Pal, 2007). Time dimension recorded in *C. versicolor* is similar in some tropical lizard species such as, *Agama impalearis* (El Mouden et al., 1999); *Phrynocephalus melanurus* (Ananjeva et al., 2006)*; Phrynocephalus horvathi* (Cicek et al., 2012). Maximum recorded longevity was also reported as 9-10 years for *Laudakia stoliczkana* (Smirina and Ananjeva, 2007), 12 -14 years for *Varanus griseus* (Smirina and Tsellarius, 1996) and 12 -13 years for *Laudakia caucasia* (Panov and Zykova, 2003).

One of the problems generally associated with skeletochronological age estimation is the phenomenon of bone resorption (Castanet et al., 1993; Smirina, 1994). In the present study the rate of endosteal resorption is assessed based on the comparison between the phalangeal sections at the same magnification from different individuals; the first innermost LAG is partially eroded in five (12.5%) individuals. There is no loss of complete LAG due to resorption unlike, earlier studied southern Indian species *H. brooki* (Pancharatna and Kumbar, 2005) and *M. carinata* (Kumbar, 2010). There is a strong positive correlation between the body mass and SVL in males ($r = 0.61$) and females ($r = 0.86$). This result suggests that body mass and size may be a reliable criterion for aging in this species.

Present data shows that males are larger than females but there is no significant difference in body mass and SVL between sexes. Similarly, the males of *Agama agama, Phrynocephalus interscapularis, Anolis opalinus* and *Cophosaurus texanus scitulus* grow larger than the females (Bellairs, 1969; Jenssen and Andrews, 1984; Shine, 1988; Sugg et al., 1995). There are some reports comparing growth rates between the sexes in lizards based on the wild populations, mark-recapture studies and maintained in outdoor terraria (Bellairs, 1969; Jenssen and Andrews, 1984; Shine, 1988; James, 1991; Sugg et al., 1995; Allan et al., 2006; Pandav et al., 2010; Cicek et al., 2012). Generally differences in SVL between male and female is caused by differences in age structure, growth rate and timing of growth deceleration (Smirina and Tsellarius, 1996; Pandav et al., 2010; Guarino, 2010). In conclusion, the results of the present study reveal that skeletochronology is applicable to tropical lizard *C. versicolor*. Animals of different body size exhibit a range from zero to five LAGs in phalanges. Moreover, there is no significant difference between body mass and SVL between the sexes for this species.

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Table 1: Body mass, SVL and number LAGs in male and female C*alotes versicolor* (Values in Mean ± SD).

Sex	Body Mass (g)	SVL (cm)	LAGs
Male	38.88 ± 17.44	9.49 ± 1.74	2.77 ± 1.31
Female	22.41 ± 9.46	8.35 ± 1.07	2.32 ± 1.04
T-value	-1.0859	1.283	- 1.0375

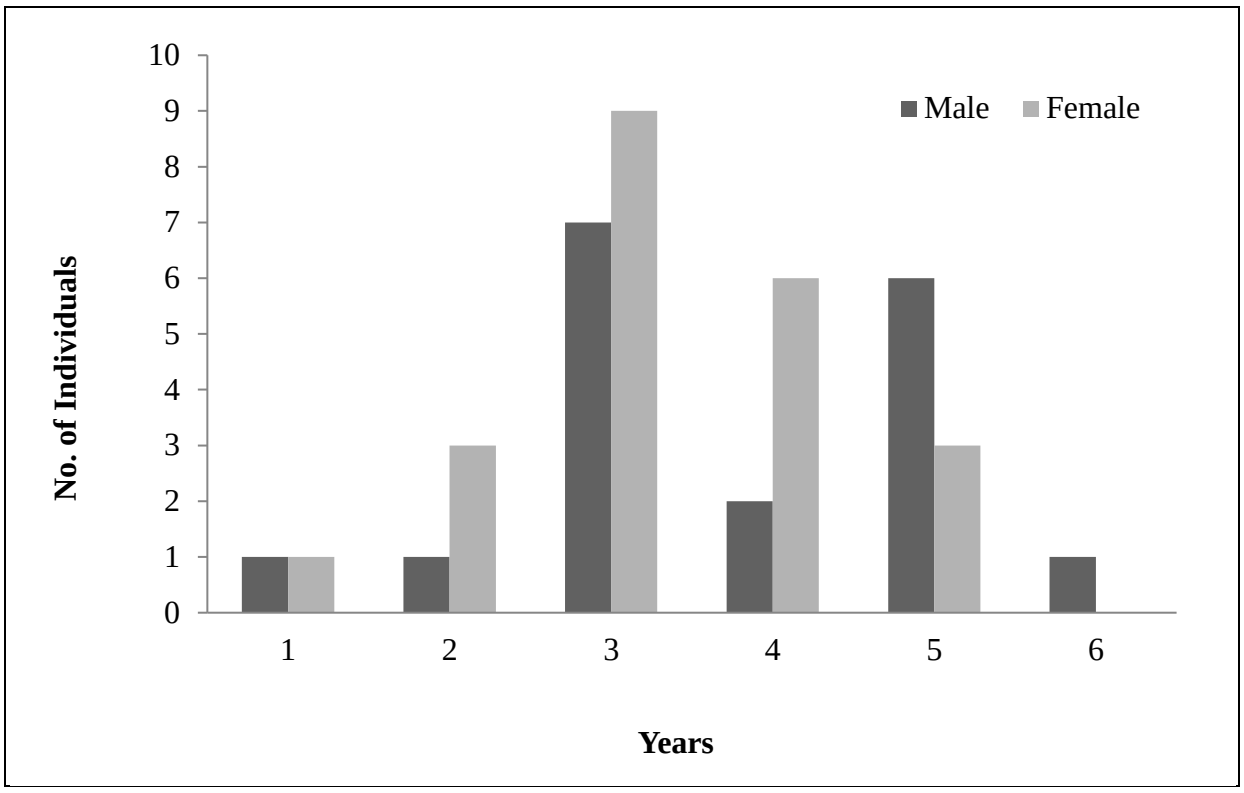
Table 2: Age, number of individuals, mean SVL and males and females SVL range of *C. versicolor*

Sex	Age	N	SVL		
			Mean	SD	Range
Female	I	01	8.4	-	-
	II	03	7.53	1.30	6.5-9.0
	III	09	8.43	0.88	7.3-10.1
	IV	06	8.26	1.02	6.5-9.5
	V	03	9.13	1.67	7.2-10.2
	Total	**22**			
Male	I	01	8.1	-	-
	II	01	11.0	-	-
	III	07	8.91	1.68	6.5-12.0
	IV	02	8.0	0.28	7.8-8.2
	V	06	10.65	1.75	8.4-13.5
	VI	01	9.5		
	Total	**18**			

Table 3: Longevity of some tropical lizard species assessed by means of skeletochronology

Sr. No.	Genus & Species	Authors	Longevity in years
1	*Hemidactylus brooki*	Pancharatna and Kumbar, 2005	4
2	Male *Calotes versicolor*	Patnaik and Behera, 1981	5
3	*Mabuya carinata*	Kumbar, 2010	5
4	*Sitana ponticeriana*	Rath and Pal, 2009	6
5	*Psammophilus dorsalis*	Mahapatro et al., 1989	5
6	*Agama impalearis*	El Mouden et al., 1997	5
7	*Phrynocephalus melanurus*	Smirina and Ananjeva, 2007	5
8	*Phrynocephalus horvathi*	Cicek et al., 2012	5
9	*Lacerta agilis*	Guarino et al., 2010	3-4
10	*Varanus griseuswas*	Smirina and Tsellarius, 1996	12-14
11	*Laudakia caucasia*	Panov and Zykiva, 2003	12-13
12	*Laudakia stoliczkana*	Ananjeva et al., 2006	9-10

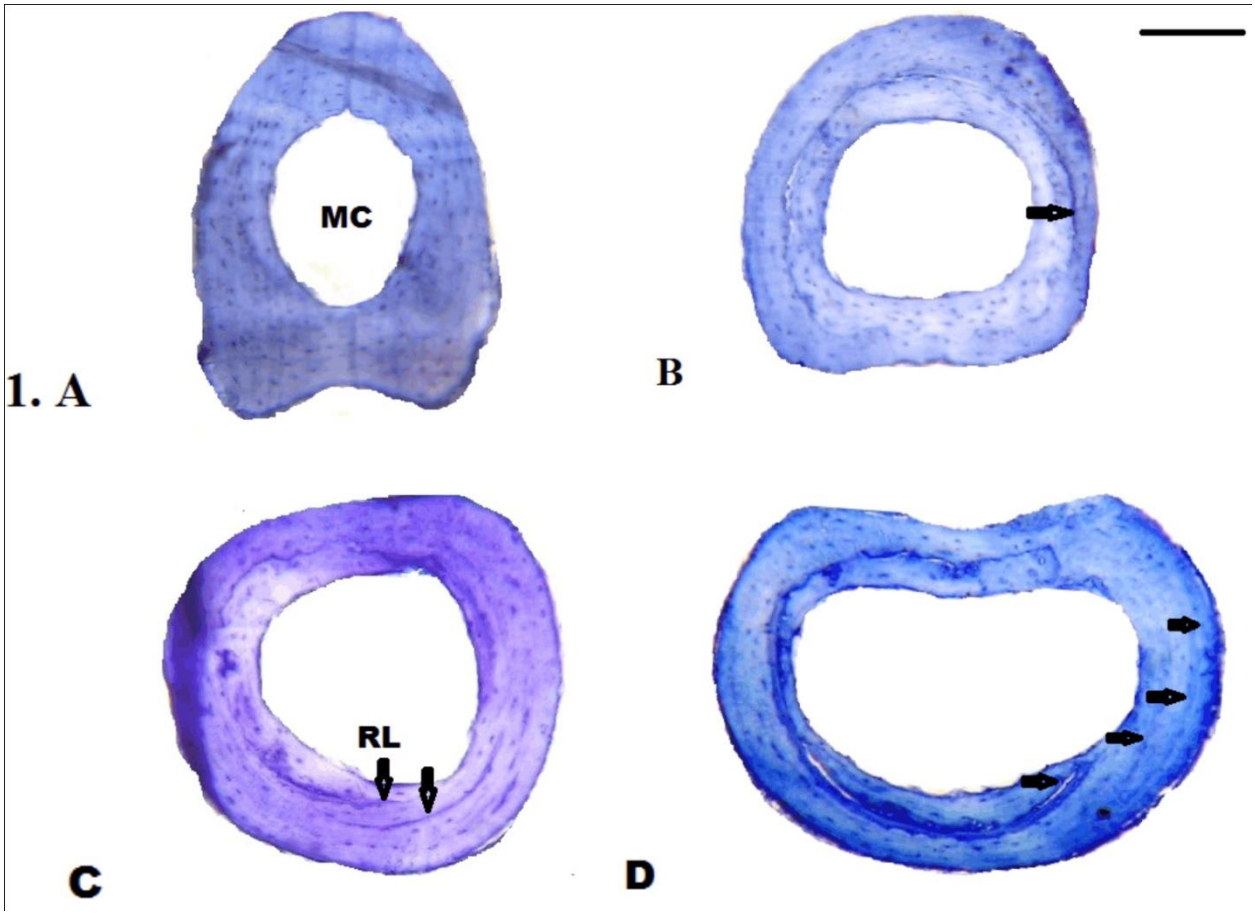
Figure 2: Number of individuals with their respective body size (cm) and number of Growth marks in the phalangeal cross section of male and female *C. versicolor*.



Explanations to Plate

Fig. 1A - D: Mid-diaphyseal **c**ross sections of right phalanges of *C. versicolor* (Hematoxylin). ***A***, Showing the absence of LAG in a female lizard with SVL 8.4 cm; ***B***, one LAG in the phalange of male lizard with SVL 9 cm; ***C***, two LAGs with resorbed line (RL) in the phalanges of female individual with SVL 8.5 cm; D, four LAGs (arrows) in the male adult with SVL 10.5 cm; Scale line = 100 µm.

Abbreviations: MC, Marrow cavity; RL, Resorption line; Arrows = Lines of arrested growth (LAGs).


MC
1. A
B
RL
C
D

Topic - V

Age can estimate in Indian bird, Red vented Bulbul *Pycnonotus cafer* by Skeletochronology

Abstract: Cyclical growth marks in cortical bone, deposited before attainment of adult body size, reflect osteogenetic changes caused by annual rhythms and are a general phenomenon in non avian ectothermic and endothermic tetrapods. Age was determined for the first time by enumerating the number of growth marks present in the cross sections of phalanges of the Indian bird Red vented Bulbul *(Pycnonotus cafer)* inhabiting southern India. Mid-diaphyseal sections of phalanges exhibited growth rings, each ring consisting of a broader growth zone and a chromophilic line of arrested growth (LAG). One to four growth marks were observed in red vented bulbul.

Key words: Bird, tropics, age, skeletochronology

Introduction: The Red-vented Bulbul (*Pycnonotus cufer*) is a common resident in India, Pakistan, Burma and Sri Lanka (Dhondt, 1977). As in its region of origin, the bulbul is common in gardens, villages and plantations, but rare along forest edges. The description of this beautiful bird is given in numerous books of English and local ethnic literature. The generalized information, on shape, size, color of eggs etc. has been described by various workers (Vijayan, 1980; Baker, 1932; Ali and Ripley, 1971). Information of pair formation and behavior of bulbul has been documented (Dixit, 1963; Lamba, 1968; Prajapati, 2006). Reproductive behavior and breeding activities of bulbul from Gujrat and Hariyana has been elaborately described (Prajapati et.al. 2011; Sharma and Sharma, 2013). Identification of the age and sex of a bird can be important for studying many aspects of avian ecology and evolution, including life history, reproductive ecology, and behavioral ecology (Jackson, 2005). Different criteria such as plumage colouration, molt, feather shape, feather wear, body size, skull ossification and iris colour have been used to estimate sex and age of bird species (Dhondt, 1977; Jackson, 2005). A lot of studies have been carried out in various fields of Red vented bulbul. Avian population studies rely heavily on knowledge of the age structure in order to calculate breeding success, survival rates and to understand the turnover of individuals within a population (Boddy, 1993; Jackson, 2005). However, there is no information on the age structure of tropical bird species using bone growth marks (skeletochronology). Hence, the present study is attempting to detect the presence of growth marks if any in the phalangeal bones of Red vented Bulbul *P. cafer* inhabiting tropical region.

Materials and methods: Red vented Bulbul is very common bird in the Sangli district compared to other bird species. Four vehicular dead Red vented Bulbul were collected from the Sangli District (16’ 75º N and 73’ 70º E), Maharashtra, India. One was collected from the Tasgaon

Chinchani road, second was collected from Tasgaon Mane-Rajuri, third was collected on Palus Devarashtre road and fourth one was collected on Palus Amnapur road. Study area receives an annual rainfall about 300–500 mm from June - October and frequently undergoes drought condition. It has dry deciduous and mixed type forest. The maximum recorded temperature was up to 41°C during summer and a minimum temperature of 10°C in winter (Kumbar and Patil, 2011). Dead specimens of Red vented Bulbuls were collected in the polythene bag and transported to the laboratory where their body size (cm) was recorded. The fourth toe was clipped from the both right and left hind limb and fixed in 10% formalin. Digits were washed in water for 1-2 h and demineralized in 5% nitric acid. Then they were washed overnight in water to remove traces of formalin and nitric acid and then preserved in 70% alcohol before processing for paraffin embedding. The transverse sections (10 µm thick) of the distal phalanx were cut on a rotary microtome (Model GE-70) and stained with Harris hematoxylin. The sections were observed under a compound microscope (Olympus CX-41) for the presence of growth rings, which were enumerated when present.

Results: Mid-diaphyseal cross sections of the phalanges of Red vented Bulbul *P. cafer* showed central bone marrow cavity surrounded by an inner narrow endosteal layer and outer relatively broad periosteal bone layer (Fig. 1A-C). In the periosteal layer a series of thin darkly stained chromophilic lines separated by wider light purple rings with sparsely distributed osteocytes were seen; the former were interpreted as lines of arrested growth (LAGs) and the latter as growth rings in the phalanges of Red vented Bulbul. One to four LAGs were equally distributed in the periosteal layer of red vented bulbul (Figs. 1A-C).

Discussion: It is well known that the age and sex of a bird can affect its migration, moult strategies, survival rates, and even foraging and roosting behavior (Ginn and Melville, 1983;

Dougall and Appleton, 1989; Gorney and Yom Tov, 1994; Dougall, 1996; Tree, 2000). Determination of age in avian species is a fundamental for a proper understanding of species biology (Boddy, 1993; Jackson, 2005). Avian population studies rely heavily on knowledge of age structure in order to calculate breeding success, survival rates and to understand the turnover of individuals within a population (Boddy, 1993). The use of lines of arrested growth (LAGs), periodically laid down in bones, is one of the best ways to obtain the age of individuals (Castnet et al. 2004). Cyclical growth marks in cortical bone reflect osteogenetic changes caused by annual rhythms and are a general phenomenon in herpetofauna and endothermic tetra-pods (Castnet et al. 2004; Turvey et al. 2005). Counting of cyclical growth marks in bones of modern birds has been restricted to the very few species for example, one living parrot, *Amazoan amazonica*, the extinct *Eocene Diatryma*, New Zealand Kiwi (*Apteryx australis*) and New Zealand moa (Bourdon et al. 2009; Ricqles et al. 2001). Although, formation of bone growth marks in these temperate species is a result of very distinct seasons or annual temperature fluctuations, in tropics LAGs are laid down during rainy months that coincide with the breeding activity (Kumbar and Pancharatna, 2001a). The results of the present study demonstrate that growth marks comparable to those found in temperate avian species are also detectable in the phalanges of southern Indian bird Red vented Bulbul. This bird usually has an extended breeding season; breeding mostly coincides with the monsoon (Sharma and Sharma, 2013; Zia et al. 2014; our personal observation). Although, environmental factors are believed to favour continuous growth, many birds inhabit the Indian continent exhibit marked seasonality in the gametogenetic and reproductive activity (Marti et al. 1979; Santhanakrishnan et al. 2011; Sharma and Sharma, 2013) suggesting that the bone growth is a cyclical phenomenon leading to the formation of LAGs even in tropical species. Further, detailed study is essential to confirm the attainment of

sexual maturity, actual breeding period and which factor is responsible for the formation of growth marks in tropical birds.

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Table 1: Body size (cm) and number of lines of arrested growth (LAG) in the cross section of phalanges of Indian Red Vented Bulbul

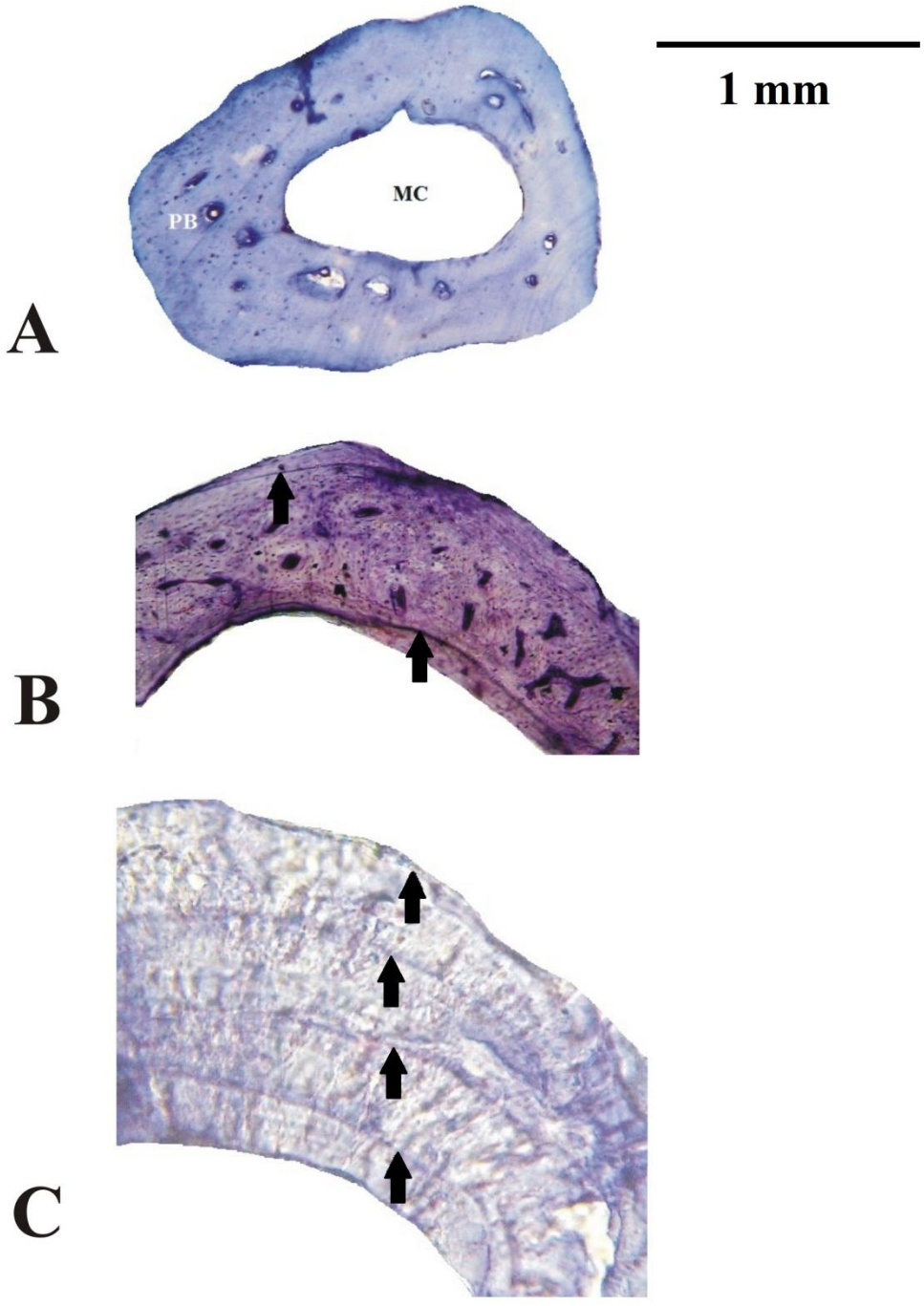
R. No.	Individuals	Body length (cm)	LAGs
1.	Red vented Bulbul	15.1	0
2.	Red vented Bulbul	15.9	2
3.	Red vented Bulbul	15.4	2
4.	Red vented Bulbul	16.5	4

Explanations to Plate

Fig. 1A - D: Mid-diaphyseal **c**ross sections of phalanges of Red Vented Bulbul (Hematoxylin). ***A***, Showing the No LAG; ***B***, two LAGs in the phalange of Bulbul with SVL 15.9 cm; ***C***, four LAGs in the phalanges of Bulbul with SVL 16.5 cm; Scale line = 100 µm.

Abbreviations: MC = Marrow Cavity; PL = Periosteal Layer; Arrows = Lines of Arrested Growth (LAGs).

PLATE - 1



[VI]

Occurrence of Growth Marks in the Phalanges of the Indian Black rat, *Rattus rattus* (Lannaeus, 1758)

Abstract: Generally vertebrate age was determined by growth layers found in the cementum in the dentine. Age was determined for the first time by enumerating the number of growth marks present in the cross sections of phalanges of the Indian black rat (*Rattus rattus*) inhabiting southern India. Mid-diaphyseal sections of phalanges exhibited growth rings, each ring consisting of a broader growth zone and a chromophilic line of arrested growth (LAG). One to five growth marks were observed in specimens with different body sizes. A strong positive correlation was also evident between body size and LAGs.

Key words: Rat, age, skeletochronology, tropics

Introduction: Age composition is one of the life history parameters needed to assess the dynamics of species populations and their management (Klevezal, 1996). Different criteria such as eye lens weight, degree of closure of cranial sutures, tooth wear and the number of corpora albicantia have been used to estimate physiological age in some mammalian species (Scheffer and Myrick, 1980). For instance, in the majority of marine mammals age has been determined on the basis of presence of growth zones in the whole teeth or sections (Cowan, 1940; Van Beneden and Gervais, 1880; Boschma, 1938; Chapskii, 1941; Scheffer, 1950; Laws, 1952; Nishiwaki and Yagi, 1953). Further, age of many temperate mammals (both terrestrial and aquatic) such as rodents, lagomorpha, carnivores, marsupials, edentata, chiroptera and primates has been estimated by counting the lines of arrested growths (LAGs) in the jaw bones and the diaphysis of long bones (Morris, 1970; Morris, 1972; Ohtaishi et al., 1976; Frylestam and Schantz, 1977; Fiala, 1978; Klevezal and Fedyk, 1978; Kovacs and Ocsenyi, 1981; Watts and Gaskin, 1932; Puzachenko, 1991; Garlich-Miller, 1993; Burke and Castanet, 1995; Castanet et al., 2004) and the results compared with those obtained from tooth cementum (Petersen and Born, 1982; Quere and Pascal, 1983). However, skeletochronological studies using bones of mammals are scarce in comparison to such studies in herpeto-fauna (Castanet and Smirina, 1990; Esteban et al., 1996; Smirina, 1994; Castanet, 2002; Kumbar and Pancharatna, 2001a; Kumbar, 2010; Kumbar and Lad, 2017). Moreover, there is no information on the age structure of tropical mammals using skeletochronology. Hence, the present study is attempted to detect the occurrence of growth marks in the phalangeal bones of the Indian black rat, *Rattus rattus* inhabiting southern India using skeletocronology.

Materials and Methods: Specimens of black rat, *Rattus rattus* (SVL = 5.5-30.2 cm; N = 21) were collected from the houses of Palus (16’ 75º N and 73’ 70º E), Maharashtra, India between

June 2013 – June 2016. Subsequently, the animals were transported to the laboratory where their body mass (g) and body size (cm) were recorded. The animals were anaesthetized using light diethyl ether. The fourth (longest) toe was clipped from the right hind limb and fixed in 10% formalin. The wound was washed, cleaned with dettol and nebasulph was applied. The rats were kept under observation until their recover and then allowed to release. Digits were washed in water for 1-2 h and demineralized in 5% nitric acid. They were washed overnight in water to remove traces of formalin and nitric acid and then preserved in 70% alcohol before processing for paraffin embedding. The transverse sections (10 µm thick) of the distal phalanx were cut on a rotary microtome (Model GE-70) and stained with Harris hematoxylin. The sections were observed under a compound microscope (Olympus CX-41) for the presence of growth rings, which were enumerated when present.

Results: The hematoxylin stained sections of the phalanges showed central bone marrow cavity surrounded by an inner narrow endosteal layer and outer relatively broad periosteal bone layer (Fig. 1A-D). In the periosteal layer a series of thin darkly stained chromophilic lines separated by wider light purple rings with sparsely distributed osteocytes were seen; the former were interpreted as lines of arrested growth (LAGs) and the latter as growth rings in the phalanges of *R. rattus*. One to four LAGs were equally distributed in the periosteal layer (Fig. 1B-D). Correlation coefficient analysis showed a positive correlation between body mass and body size ($r = 0.87$) as well as body size and the number of LAGs ($r = 0.70$).

Determination of age, reproduction and longevity of the marine mammals is most essential for stock assessment and proper management. The use of lines of arrested growth (LAGs), periodically laid down in teeth and skeletal tissues, is one of the best ways to obtain the age of individuals. As suggested by many authors, study of species from different habitats may

help to disentangle the direct effects of environment from intrinsic rhythms (Sergeant, 1967). Experimental evidence indicates that the formation of growth marks in the teeth and skeletal tissues in the different regions is controlled by different physiological factors such as nutrition, breeding activity, intrinsic rhythms and photoperiod (Cowan, 1940; Castanet et al., 2004; Klevezal and Kleinenberg, 1969). In many tropical and temperate amphibians and reptiles, cyclical pattern of bone growth has been well established (Smirina, 1994; Pancharatna, 2002; Kumbar and Pancharatna, 2001b). Although, formation of bone growth marks in temperate species is a result of annual temperature fluctuations (Castanet and Smirina, 1990; Esteban et al., 1996; Smirina, 1994), in tropics LAGs are laid down during rainy months that coincide with the breeding activity (Castanet and Smirina, 1990; Kumbar and Pancharatna, 2001a; Kumbar and Pancharatna, 2001b; Kumbar and Pancharatna, 2004). The results of the present study demonstrate that growth marks comparable to those found in amphibians and reptiles are also detectable in the phalanges of southern Indian black rat, *Rattus rattus*. Although, environmental factors are believed to favour continuous growth, many rodents inhabiting the Indian peninsular exhibit marked seasonality in the growth pattern, gametogenetic and reproductive activity (Chauhan and Saxena, 1985a; Chauhan and Saxena, 1985b; Vadell et al., 2010), suggesting that the bone growth is a cyclical phenomenon leading to the formation of LAGs even in tropical species. Further, the positive correlation between body mass and length and body length and LAGs in *R. rattus* indicate that larger individuals have experienced greater number of growth cycles and hence, may be older. Further, detailed study is essential to confirm whether or not the growth marks are formed annually and hence can be used as ‘year rings’ for estimating age and longevity in tropical mammals.

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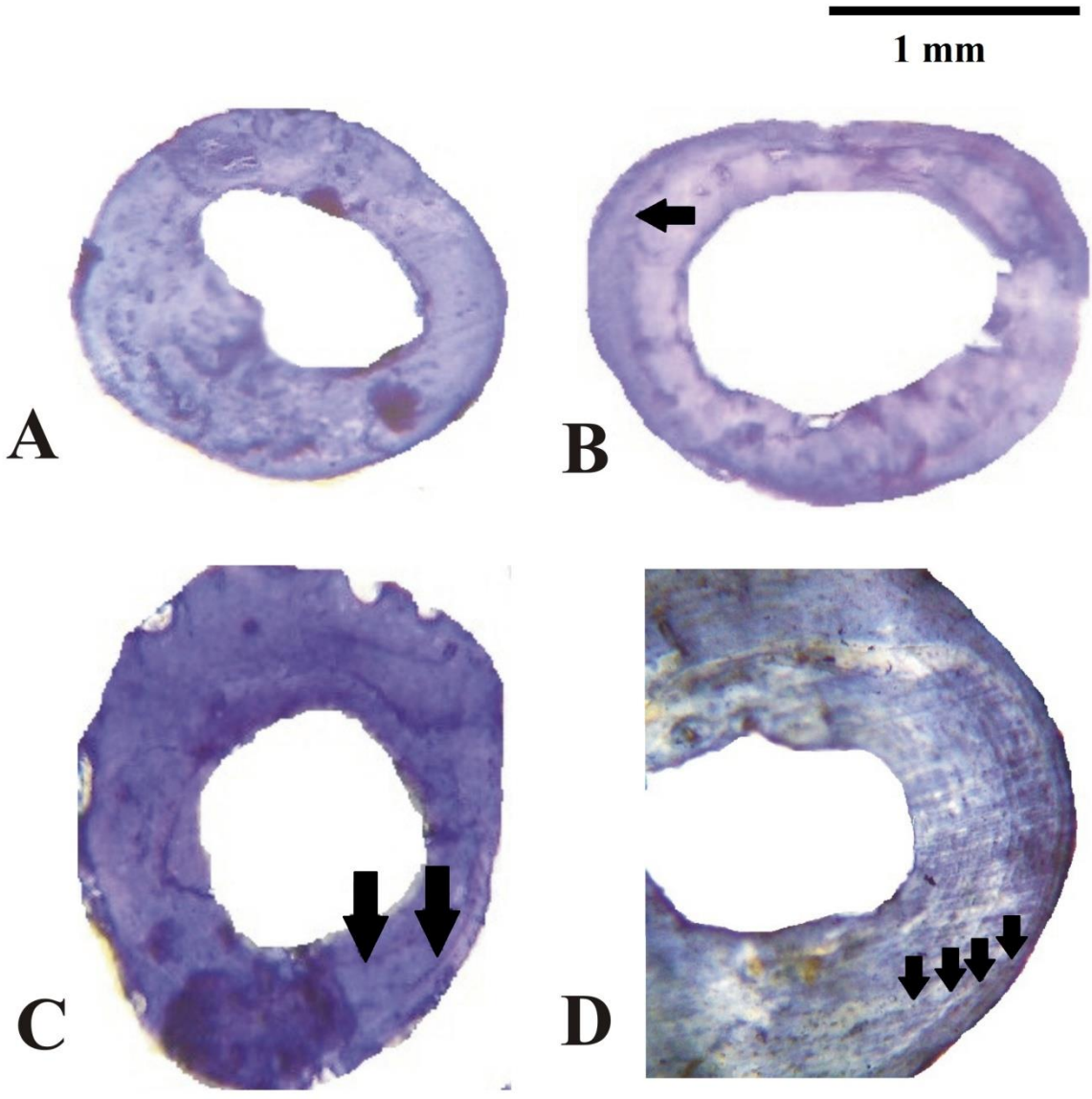
Explanations to Plate

Fig. 1A - D: Mid-diaphyseal **c**ross sections of right phalanges of *Rattus rattus* (Hematoxylin). ***A***, Showing the absence of LAG in the rat with SVL 7.5 cm; ***B***, one LAG in the phalange of rat with SVL 14 cm; ***C***, two LAGs in the phalanges of rat with SVL 28.2 cm; ***D***, four LAGs (arrows) in the rat with SVL 30.2 cm; Scale line = 100 µm.

Abbreviations: MC = Marrow Cavity; PL = Periosteal Layer; Arrows = Lines of Arrested Growth (LAGs).

PLATE 1



PUBLISHED RESEARCH PAPERS

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Comparison of Otolith, Scale and Vertebrae for Age Estimation in Freshwater Exotic Fish *Oreochromis mossambicus*

KEYWORDS: Comparison, age, longevity, exotic fish, *Oreochromis mossambicus*

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ABSTRACT *Otolith, scale and vertebrae sections of freshwater exotic fish O. mossambicus (N = 133) were compared to ascertain the best aging material. Different body sized fishes revealed the presence of 1 - 7 growth marks. Agreement between otolith and scale ages was 96.96% and otolith and vertebrae ages were 88.36% in 33 fishes used for comparative studies. Vertebrae ring counts (VRC) were under estimated the age in (13.6%) older individuals compared to otolith and scale ring counts (SRC). The results revealed that otoliths and scales are reliable calcified materials for estimating age compared to vertebrae in O. mossambicus. There was a highly positive correlation between body length (BL) and body weight (BW). Further, otoliths ring count (ORC), otoliths weight (OW) and otoliths diameter (OD) showed positive correlation with BL and BW. This fish may live for a maximum of 7 years in the natural population.*

INTRODUCTION

Freshwater exotic fish *O. mossambicus* was noticed in the fish catch, probably on account of accidental entry with Indian major carp seed in 1990s. This fish population has been continuous increasing in the total number of caught in Indian major Rivers (Jain and Gupta, 1994) which cause of worry for the fishery biologists (Anon, 1995; Biju Kumar, 2000). Moreover, as per 2006 Global Invasive Species Database *O. mossambicus* is listed being as in the top 100 invasive alien species on the planet (Canonico *et al.*, 2005). Invasive populations are now causing environmental and ecological problems in many countries including India (Canonico *et al.*, 2005). *O. mossambicus* is widely distributed and now forms a part of fish fauna in the Godavari, Krishna, Cauvery, Yamuna and Ganga Rivers (Lakra *et al.*, 2008).

Growth and age studies provide important demographic parameters to analyze and asses fish populations (Maceina & Sammons, 2006). Ageing of fishes from tropical regions have been reported through annual increments in calcified structures such as, scales (Werder and Soares, 1985; Mayekiso & Hecht, 1988; Sudarshan & Kulkarni, 2013), dorsal and pectoral spines (Bio & Ikusemiju, 1981; Pantulu, 1961), vertebral centra (Brown & Gruber, 1988; Bahuguna, 2013), and otoliths (Fowler & Doherty, 1992; David & Pancharatna, 2003). Most of the earlier age estimation studies of Indian fishes have been concentrated on scales (Singh & Sharma, 1998; Dua & Kumar, 2006; Kanwal & Pathani, 2011; Ujjania *et al.*, 2013) due to the easiest to collect, process and avoids sacrificing the specimens. Recent reviews have suspected on scale aging due to difficulties in reading annuli, low precision (Lowerre-Barbieri *et al.*, 1994), and that scale ages may become inaccurate when growth becomes asymptotic (Beamish & McFarlane, 1987; Shepherd, 1988). Scales have generally been found to underestimate ages relative to other structures, especially for older individuals and in slow-growing populations (Campbell & Babaluk, 1979; Mills & Beamish, 1980; Erickson, 1983; Kocovsky and Carline, 2000). Since scale growth is assumed to be proportional to body growth (Whitney & Carlander, 1956; Hile, 1970; Bagenal, 1974; and Erickson, 1983), annuli become crowded on the scale edges in slow-growing populations and in older fish, making scale interpretation difficult. To overcome from these difficulties, comparison of various calcified materials have been performed in many species, including yellow perch *Perca flavescens* (Niewinski & Ferreri, 1999), river carp suckers *Carpiodes carpio* (Braaten *et al.*, 1999), and white suckers *Catostomus commersonii* (Scidmore & Glass, 1953; Ovchynnyk, 1969; Quinn & Ross, 1982; Sylvester, 2006). Although, some studies are also available on age and growth of *O. mossambicus* by using scales (Ujjania *et al.*, 2013) and otoliths (Booth & Merron, 1996; Panfili & Tomas, 2001). There were no comparative studies for selection of reliable calcified material for aging in this species. Present investigation aims to select the most reliable calcified material for estimating the age of exotic freshwater fish *O. mossambicus* inhabiting Krishna River, Southern India.

MATERIALS AND METHODS

Different body sized freshwater fishes (*O. mossambicus*, N = 133) were collected from the Krishna River, Sangli District, Maharashtra, India with the help of local fisherman and purchased from local market in 2012 - 2013. Total body length (BL) of each fish was measured from the tip of snout to tip of the caudal fin (in cm). Body weight (BW) was recorded by using single pan balance nearest to 0.01gm, simultaneously lateral line scales, otoliths and vertebrae of each specimen was collected and preserved in different vials for further studies.

Scale study:

Lateral line scales of each specimen was collected with the help of forceps, cleaned in water by rubbing gently with fingers, fixed in 10% formalin solution for 24 hours and washed in water for 2 hours. Cleaned scale was kept in between two clean slides and slides were tied with rubber band on either side and observed under binocular microscope (Magnus MSZ-BI) for enumerating the ring counts present on the scales and photographed by digital camera (ABBOT DEC-2000).

Otolith Studies:

Otoliths were collected by taking an incision on the dorsal side of the head, to expose the brain on either side of which the otic capsules are located. The sagittal otoliths were removed from the otic capsules by opening the otic bulla. Both sagittae were retrieved intact from each specimen, washed in water and cleaned from all extraneous tissue. Then, each otolith was weighed to the nearest 0.001 mg the diameter was measured to the nearest 0.01 mm using a standard micro screw gage (Newman *et al.*, 2000). Otoliths were then immersed in 50% glycerol and observed under binocular microscope (Magnus MLXB). Growth rings were clearly visible as alternate opaque and translucent zones that were enumerated.

Volume : 4 | Issue : 6 | June 2014 | ISSN - 2249-555X

Vertebrae Section:
Different body sized 33 (BL 6.4 – 35.5 mm) fishes were used for vertebrae sections. Central 5th and 10th vertebrae of each fish were excised, cleaned and fixed in 10% formaldehyde solution for 24 hours, then washed in water for 1 hour and decalcified with 10% nitric acid (duration of decalcification depends on size of vertebrae). Decalcified vertebrae were washed under running tap water for 24 hours to remove the traces of formaldehyde and nitric acid and preserved in 70% alcohol. These vertebrae were embedded in paraffin wax and sectioned (10µ thickness) by using a rotary microtome (Model GE-70). Mid-diaphyseal sections were stained with Harris haematoxyline and observed under compound microscope for enumeration of growth marks (Buckmeier *et al.*, 2002).

Comparison of otoliths, scales and vertebrae sections of 33 fishes were undertaken for the selection of reliable calcified material for accurate assessment of age and longevity. For each fish, growth marks were counted on the aging structures independently by two readers without prior information of body length and body weight. Then percent agreement was calculated between otolith ring counts with scales and vertebrae ring counts.

Statistical analysis:
The relationship if any, between BL and BW, BL and OW, BL and OD, BW and OW, BW and OD was assessed by plotting scatter diagrams and using regression analysis (Zar, 1984). The relationship between ORC and BL, ORC and BW, ORC and OW and the ORC and OD was determined by calculating the correlation coefficient '*r*' by Karl Pearson's method (Zar, 1984).

RESULTS

Monthly variation in ambient temperature and rainfall in the year 2012 - 2013 is shown in figure 1. Although the mean monthly temperature varied between 21 – 30°C and that of rainfall from 0 to 121 mm (Fig. 1)

The otoliths, scales and vertebrae of *O. mossambicus* showed annual rings, each ring composed of a faint broader growth zone and a dark opaque line (Lines of arrested growth) the LAG (Plate 1A-F). One to seven growth marks were observed in the otoliths, scales, and vertebrae of different body sized fishes (N = 133). Out of 133 fishes, 21 (15.79%) fishes with mean body length (13.9 cm) have not exhibited any LAG (Table 1, Plate 1A, C, E). Thirty fishes (22.56%) with mean body length (16.28 cm) showed 1 LAG (Table 1), 38 fishes (28.57%) with mean body length (16.71 cm) exhibited 2 LAGs, 29 fishes (21.80 %) with mean body length (15.57 cm) showed 3 LAGs, 8 fishes (6.02%) with mean body length (17.21cm) exhibited 4 LAGs, 4 fishes (3.00 %) with mean body length (18.95 cm) showed 5 LAGs and 3 fishes (2.26%) with mean body length (23.66 cm) exhibited 6 LAGs (Table 1 & Plate 1B, D, F). It was generally observed that in older fish growth marks appeared sharp at the periphery of the otoliths, scales, and vertebrae sections (Plate 1B, D, F).

Comparison of otolith ring count with scale and vertebrae ring count in 33 large body sized fishes were done, the number of rings remained identical in otoliths, scales and vertebrae sections of 26 (78.78%) fishes. In one (3.03%) fish, the scales showed short on one growth ring compared to otolith in 3rd year individual. In 4 (12.12%) fishes, the vertebrae showed one growth ring lesser compared to otolith rings in 3rd year age groups and 2 (6.06%) fishes were showed short on two growth rings in 4th and 7th year age group individuals.

Of the 133 fishes sampled, the body length (BL) ranged from 6.0 – 35.5 cm with an average of 20.75 cm and body weight (BW) varied between 5 and 760 g with a mean of 382.5 g (Table 1). There was a high degree of positive correlation (r = 0.736) between BL and BW (Fig. 2). The scatter diagrams indicated that BL correlated highly positively with OD (r = 0.841; Fig. 3) and OW (r = 0.647; Fig. 4). Further, BW also showed positive correlation with OD (r = 0.628; Fig. 5) and OW (r = 0.759; Fig. 6). While, the ORC showed a lesser correlation with BW (r = 0.447), BL (r = 0.220), OW (r = 0.273) and OD (r = 0.144).

DISCUSSION

Age and growth studies of fish provide information of rate of survival, growth, mortality, age composition, and reproduction rate (Polat *et al.*, 2001; Campana, 2001). In the present study, one to seven broader light growth zones and one to six opaque sharp growth-arrested zones were found in fishes of varying body size (Table 1 and Plate 1). The comparative studies of otolith, scale, and vertebrae of freshwater exotic fish *O. mossambicus* revealed the presence of growth marks similar to that reported for other tropical species namely, *Sillago japonica* (Sulistiono *et al.*, 1999); Indian whiting, *S. indica* (David & Pancharatna, 2003); *Catla catla* (Ujjania, 2012); and *Notopterus notopterus* (Sudarshan & Kulkarni, 2013). The meteorological data indicate that, the annual temperature variation of Sangli is within 9°C, and the rainfall exhibited considerable annual variation (Fig. 1), further, during monsoon season there were increased temperature and maximum rainfall, consequently, cyclic pattern of growth in hard calcified material is common in fishes which is generally attributed to enforced seasonal feeding activity owing to marked variations in surrounding climatic factors, e.g. the extreme seasonal shifts in temperature of temperate areas, and the typical wet and dry conditions of the humidity cycle in the tropics (Johal & Tandon, 1992; Seshappa, 1999; Campana, 2001). Most of the freshwater fishes inhabiting Southern India exhibits a clear seasonality in body growth, breeding activities and gametogenesis (Saidapur *et al.*, 1989). Even though, *O. mossambicus* is spawning in July – August (rainy season) when spawning of most of the Indian major carps (*C. catla*, *L. rohita*, and *C. mrigala*) occurred (Ganie, 2013). Similar observations on the other fishes have reported (Seshappa, 1958; 1972; Alam & Pathak, 2010). Moreover, experimental studies have been confirmed that the formation of growth marks are annual in many species including, *Cirrhinus mrigala* (Hanumantha Rao, 1974), *O. niloticus* (Abdel-Hadi *et al.*, 2000); *Clarias gariepinus* (Wartenberg *et al.*, 2011), *Puntius conchonius* (Bahuguna, 2013); and *Labeo rohita* (our personal observation). Therefore, number of growth marks present in scale, otolith and vertebrae section is directly depict the age of individual fish.

Among 133 fish samples, 21 fishes showed no LAG in its calcified materials indicating that the fishes may be in their first year of growth. One to third year age group individuals are dominant with varied body length (6.0 – 35.2 cm). Further, the positive correlation between body length and growth mark of these fishes indicate that larger individuals have experienced a greater number of growth marks and therefore may be older. Larger fishes exhibited increased number of growth marks, thus there was a positive correlation between OW, OD with BL and BW, whether the body length and weight influences age needs further elucidation.

Comparison of the three ageing structures within the current study revealed that otolith is the most precise material for age estimation in *O. mossambicus* due to clear and distinctness in growth marks. The suitability of otolith for age estimation is also supported by several workers (Seshappa, 1999; Campana, 2001) the fact that otolith do not show reabsorption and their growth is acellular rather than by calcification (Secor *et al.*, 1995) and also because otoliths are reported to be metabolically inert and thus do not reflect physiological changes that may occur throughout the life of fish (Phelps *et al.*, 2007). Otoliths continue to grow and form annuli even as body growth slows and asymptotic length is reached, and annuli reasbsorption does not appear to occur during periods of food limitation or stress (Devries & Frie, 1996). Several workers were reported that otoliths to be the most reliable ageing structure in a number of temperate as well as tropical species such as, *Chelidonichthys kumu* (Staples, 1971),

Volume : 4 | Issue : 6 | June 2014 | ISSN - 2249-555X

Capoeta capoeta umbla (Ekingen & Polat, 1987), *Trachurus trachurus* (Polat & Kukul, 1990), *Pylodictis olivaris* (Nash & Irwin, 1999), *Indian whiting* (David & Pancharatna, 2003) and *Ictalurus punctatus* (Buckmeier *et al.*, 2002; Colombo *et al.*, 2010). In the comparative studies, out of 33 fishes 32 fishes the number of growth marks was identical in scales and otoliths indicating that scale aging is reliable in > 97% of fishes. In other hand among 33 fishes 4 fisnes one number of growth marks were short and another 2 fishes two growth marks were lesser in vertebrae sections compared to otolith ring counts in older individuals. Result indicating that vertebrae ring count is not that much reliable in this fish, this might be due to overcrowding of growth marks at the periphery of vertebrae sections, which was difficult to differentiate and count.

Our study found variation in the otolith, scale, and vertebrae ages of *O. mossambicus*. In this fish, vertebrae section underestimated the ages when compared with the otolith and scale ages. However, otolith and scale ring counts are more distinct and easy to enumerate, even in the older fish, than the vertebrae ring count. Therefore, we assume that the otolith and scale provide a reliable source for aging in *O. mossambicus* inhabiting Southern India.

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Table 1 Showing number of fishes, percentage, mean body weight, length, otolith weight, diameter and number of lines of arrested growths (LAGs) and age frequencies of freshwater exotic fish *O. mossambicus* (N = 133).

Number of Fishes	Percentage (%)	Body Weight (gm)	Body Length (cm)	Otolith Weight (mg)	Otolith Diameter (mm)	No. of LAGs	Age Frequency
21	15.79	83.57	13.9	21.11	0.276	0	1st Year
30	22.56	108.9	16.28	27.53	0.350	1	2nd year
38	28.57	151.94	16.71	39.35	0.366	2	3rd year
29	21.80	179.20	15.57	39.87	0.344	3	4th year
08	6.02	314.62	17.21	64.00	0.323	4	5th year
04	3.00	249.25	18.95	70.67	0.417	5	6th year
03	2.26	394.66	23.66	39.8	0.421	6	7th year

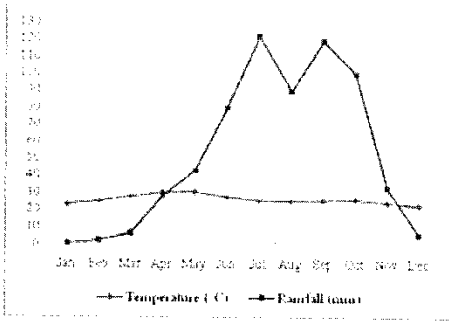


Figure 1: Figure shows monthly variation in mean temperature and rainfall of Sangli District from January 2012 to December 2013.

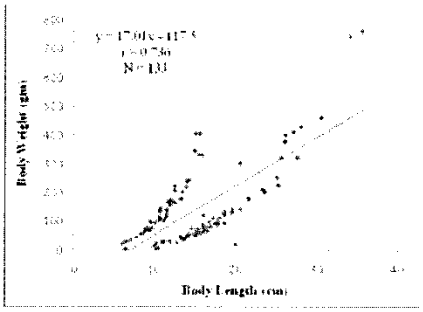


Figure 2: Relationship between Body Length (BL) and Body Weight (BW) in *O. mossambicus*.

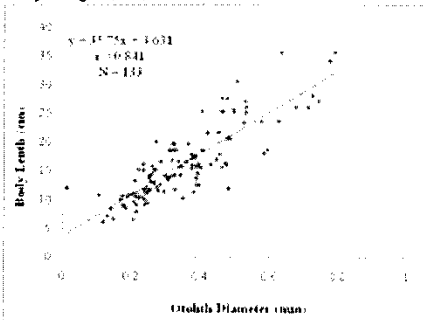


Figure 3: Relationship between Body Length (BL) and Otolith diameter (OD) in *O. mossambicus*.

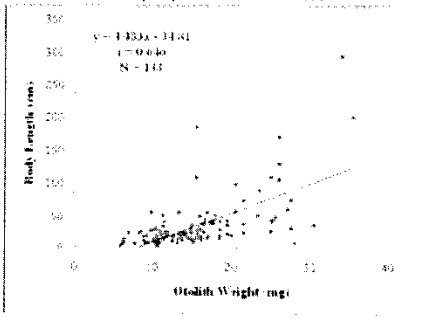


Figure 4: Relationship between Body Length (BL) and Otolith weight (OW) in *O. mossambicus*.

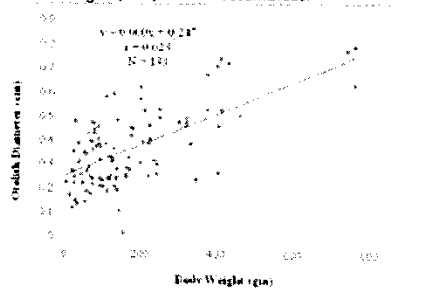


Figure 5: Relationship between Body Weight (BW) and Otolith diameter (OD) in *O. mossambicus*.

Volume : 4 | Issue : 6 | June 2014 | ISSN - 2249-555X

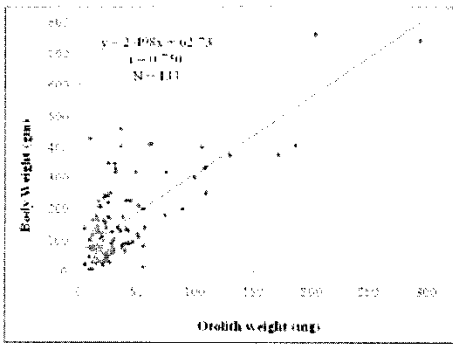
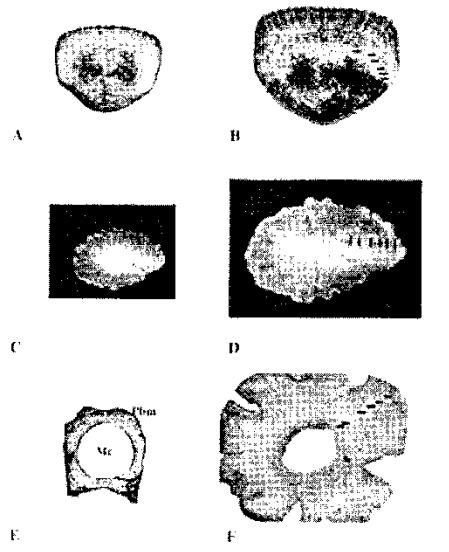


Figure 6: Relationship between Body Weight (BW) and Otolith weight (OW) in *O. mossambicus*.

PLATE I



Volume : 4 | Issue : 6 | June 2014 | ISSN - 2249-555X

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Estimation of age and longevity of freshwater fish *Salmophasia balookee* from otoliths, scales and vertebrae

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Abstract

Age and longevity of freshwater fish *Salmophasia balookee* was assessed by comparing otoliths, scales and vertebrae ring counts. One to five growth rings consisting of growth zones and lines of arrested growth (LAGs) were noticed in different body sized fishes. Out of 134 fish, 9 (6.97%) fish were in the first year growth, 31 (23.13%) in second year, 59 (44.03%) in third year, 27 (20.15%) in fourth year and 5 (5.97%) fish in the fifth year growth. Comparative study showed that scale ring counts (SRC) was under estimated the age as compared to the otolith ring counts (ORC) and vertebrae ring counts (VRC). The percent agreement between otolith and scale ages were 88.81% and otolith and vertebrae ages was 99.25%. The results of the present study indicate that otoliths and vertebrae are most suitable aging materials as compared to scales in *S. balookee*.

Key words

Age validation, Longevity, *Salmophasia balookee*

Introduction

Studies on age and longevity provide important demographic parameters to analyze and assess the fish population (Maceina and Sammons, 2006). However, obtaining accurate age information is crucial for precise understanding of these metries (Campana, 2001). Age has been determined in tropical freshwater fish through annual increments in calcified structures like, scales, spines, vertebrae and otoliths (Khan *et al.*, 2015). Scales have widely been used for determination of age in earlier studies, due to the fact that their removal is non-lethal, easy to collect and prepare (Braaten *et al.*, 1999; Kanwal and Pathani, 2011; Khan *et al.*, 2015); however, this method might not reveal the true age of slow growing and older fish (Dua and Kumar, 2006; Kanwal and Pathani, 2011; Ujjania *et al.*, 2013). Also, Khan *et al.* (2015) found that the annual rings present in scales in *H. molitrix* were not clear as compared to other structures. Indistinctness of annuli at the outer edges of scales makes them unreadable in older fish (Akombo *et al.*, 2015). Hence, other alternative calcified materials like, otoliths, spines, opercular bones and vertebrae have been used over these year as annuli are easily recognizable even in older fish than scales (Casselman, 1990). Otoliths have several advantages for estimation of age as they are not subjected to resorption, their growth is acellular rather than by ossification, and also otolith annuli are more distinct and easy to enumerate even in older fishes (Secor *et al.*, 1995; Hoxmeier *et al.*, 2001). Phelps *et al.* (2007) reported that otoliths are metabolically inert and thus do not reflect physiological changes that may occur throughout the life of fish. Otoliths grow continuously and form annuli even as body growth slows and asymptotic length is reached, and annuli reasbsorption does not appear to occur during periods of food limitation or stress (Colombo *et al.*, 2010). Hence, many studies have employed otoliths for estimating age in fish (David and Pancharatna, 2003; Weyl and Booth, 2008; Colombo *et al.*, 2010; Khan *et al.*, 2011). Even, vertebrae are also best calcified material for aging in fish (Khan *et al.*, 2011b; Bahuguna, 2013). Therefore, selection of precise calcified materials for accurate aging of fish is more challenging for fishery researcher.

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Krishna River harbours a rich diversity of fish leading to globally threatened and endemic fishes. *S. balookee* is one of the most commercial and popular food fish in this region. Presently, it is threatened by anthropogenic sources such as, accumulation of industrial, agricultural effluents, domestic organic wastes and non-degradable plastic materials owing to tourism activities. Besides, unscientific practice for collection of fishes has increased to meet high market demand. Since last decade, sand mining and bricks manufacturing activities have alarmingly increased along some stretches of the river system. If the present situation continues, the adverse conditions might lead to loss of most of fish fauna of Krishna River. Proper demographic and population dynamic study is necessary for protection and conservation of this species in the river system. There is no single report on aging of *S. balookee* inhabiting the Krishna River. Therefore, present investigation was undertaken to estimate age, longevity, and to select the most reliable calcified material from scales, otoliths and vertebrae for accurate aging of *S. balookee*.

Materials and Methods

Freshwater fish *S. balookee* ($N = 134$) were collected from the Krishna River, Sangli District (17°09' N & 74°45' E), Maharashtra, Southern India with the help of local fisherman, and were also purchased from the local fish market during the year 2014. Fish were brought to the laboratory where body length (BL) from the tip of snout to the longest caudal fin lobe (in cm) and body weight (BW), using single pan balance nearest to 0.01gm of each fish, were recorded. Simultaneously scales, otoliths and vertebrae of each fish was collected for comparative studies.

Scale study : Lateral line scales were collected with the help of pointed forceps, cleaned in water by rubbing through the fingers then fixed in 10% formalin solution for 24 hrs and then washed in water for 2 hrs. Scale was kept in between two clean slides and slides were tied with rubber band on either side and observed under binocular microscope (Magnus MSZ-BI) for enumerating the number of growth rings present on the scales, and then photographed with digital camera (AIBBOT DEC2000).

Otolith study : Otoliths were collected by making an incision on the dorsal side of the head, to expose the brain on either side of which the otic capsules are located. The sagittal otoliths were removed from the otic capsules by opening the otic bulla. Both sagittae were retrieved intact from each specimen, washed in water and cleaned from all extraneous tissue. Then, each otolith was weighed to nearest 0.001 mg and diameter of otolith was measured to nearest 0.01 mm using a caliper rule (Newman *et al.*, 2000). Otoliths were immersed in 50% glycerol and observed under binocular microscope. Growth rings were clearly visible as alternate opaque and translucent zones that were enumerated.

Vertebrae study : Central 5-10 vertebrae of each fish were excised, cleaned and fixed in 10% formalin solution for 24 hrs, then washed in running water for one hour, later decalcified with 10% nitric acid. Decalcified vertebrae were washed in running water for 24 hrs for removal of formalin and nitric acid then preserved in 70% alcohol until paraffin embedding. Vertebrae were sectioned (10 µm thick) using a rotary microtome (Model GE - 70). Mid-diaphyseal sections were stained with Harris haematoxylin and observed under compound microscope for enumeration of growth rings and photographed good sections. Growth rings in three calcified materials of each fish were counted independently by two readers without prior information of body weight and body length. Percent agreement between Otolith Ring Count (ORC) and Scale Ring Count (SRC), ORC and Vertebrae Ring Count (VRC) was calculated. Further, relationship, if any, between BL and BW, otolith weight (OW) and BL; OW and BW; otolith diameter (OD) and BW; OD and BL, and ORC and BL; ORC and BW was determined by calculating correlation coefficient 'r' by Karl Pearson's method (Zar, 1996).

Results and Discussion

In the present study, 134 fish with mean body length of 13.55 cm and mean body weight of 138.5 gm were used. All the three calcified materials showed growth rings each ring composed of faintly stained broader growth zone, and a darkly stained condensed line, lines of arrested growth (LAGs). Otolith was more or less circular in shape, thin and transparent in which clear and distinct growth rings were observed. One to four LAGs were noticed in fish of different body sizes. Out of 134 fishes, 9 fish with mean body length 10.75 ± 0.44 cm showed no LAGs in otoliths; 31 fish with mean body length 11.60 ± 1.25 cm exhibited one LAG each; 59 fish with mean body length 12.24 ± 1.92 cm exhibited 2 LAGs; 27 fishes with mean body length 13.52 ± 2.49 cm showed 3 LAGs; and 8 fish with mean body length 15.00 ± 2.81cm possessed 4 LAGs in otoliths, respectively (Table 1).

In the present study, ORC was compared with SRC and VRC for selection of most reliable calcified materials for accurate aging in this fish due to reason that otolith rings were more distinct, clear and easy to enumerate. Comparative study showed that number of growth rings were identical in otoliths and scales in 119 (88.81%) fish out of 134 (Table 2). Remaining 15 fish (11.19%) had one growth ring short in scales as compared to otoliths in different age groups. This result indicates that increasing otolith age on one side and on other side decreased percent agreement in scale age (Table 2). Vertebrae sections of *S. balookee* were circular with central marrow cavity surrounded by broad periosteal bone. Haemetoxylin stained sections showed clear and distinct

Table 1: Otolith characteristics in relation with body weight, body length in the freshwater fish *S. balookee* (N = 134)

No. of LAGs	Age	Number of fish	%	Body weight (gm)	Body length (cm)	Otolith weight (mg)	Otolith diameter (mm)
0	1	9	6.72	35.67 ± 10.99	10.75 ± 0.44	0.76 ± 0.408	1.43 ± 0.25
1	2	31	23.13	47.37 + 14.81	11.60 + 1.25	1.13 ± 0.65	1.50 ± 0.24
2	3	59	44.03	61.18 ± 34.21	12.24 ± 1.92	1.37 ± 0.84	1.54 ± 0.32
3	4	27	20.15	88.40 + 53.24	13.52 ± 2.49	1.78 ± 1.21	1.64 ± 0.34
4	5	8	5.97	115.47± 58.46	15.0 ± 2.81	2.37 + 1.34	1.86 ± 0.23

Note: Values are means + SD

Table 2: Agreement and disagreement of scales and vertebrae ages with otolith age in *S. balookee* (N = 134)

Otolith age	Number of fish	Percent agreement	Remarks
Scale Ages			
I	09	100	-
II	31	98.51	1.49(2) UE
III	59	96.27	3.73(5) UE
IV	27	95.52	4.48(6) UE
V	08	98.51	1.49(2) UE
Vertebrae Ages			
I	09	100	—
II	31	100	—
III	59	99.26	0.74(1) UE
IV	27	100	- -
V	08	98.51	1.49(2) OE

Number in parenthesis indicates that number of fish; UE = under estimation and OE = over estimation

growth rings in periosteal layers *i.e.,* lighter stained growth zone and darkly stained LAGs. One to four LAGs were observed in vertebrae sections. Comparative study showed that number of growth marks was identical in otolith and vertebrae sections in 133 (99.25%) fish out of 134 (Table 2). Further, two fish (1.49%) had an extra LAG in the periphery of periosteal layer in the four year age group individuals, but one (0.75%) fish had one LAG short as compared to otolith in three year age group (Table 2). Percent agreement between otoliths and vertebrae age was 99.25% in this fish.

Randomly collected fish for age determination indicated that 6.72% fishes were in the first year growth, 23.13% in second year, 44.03% in third year, 20.15% in fourth year and 5.97% fishes in the fifth year of growth. There was a high degree of positive correlation between BL and BW (r = 0.94; Table 3). Otolith weight was positively correlated with body length (r = 0.67) and body weight (r = 0.73) and OD with BW (r = 0.55) and BL (r = 0.55), respectively. Further, ORC also showed moderate correlation with BL (r – 0.44) and BW (r = 0.47) (Table 3).

The presence of one to five growth rings in scales, otoliths and vertebrae sections of freshwater fish *S. balookee* inhabiting Sangli District showed similar to that reported in other tropical species namely, *Sillago japonica* (Sulistiono *et al.*, 1999), Indian whiting, *Sillago indica* (David and Pancharatna, 2003), *Catla catla* (Ujjania, 2012) and *Notopterus notopterus* (Sudarshan and Kulkarni, 2013). The fact that most of the Indian freshwater fish exhibit seasonal variation in body weight, gametogenic and breeding activity (Alam and Pathak, 2010; Ganie *et al.,* 2013), it suggests that formation of growth rings may be a cyclical phenomenon leading to the formation of LAGs. Therefore, the cyclic pattern in growth rings in *S. balookee* might be due to the annual rainfall pattern which in turn might affect regular feeding activity by diverting the fish towards reproductive activity. Similarly, Akombo *et al.* (2015) attributed that the formation of growth marks on hard structures in tropical fishes as a result of reproductive activity, feeding intensity, lower salinity, increased turbidity and reduced temperature during rainy season in water bodies. Furthermore, variation of water temperature might have impact on deposition of calcium materials in otoliths and other calcified structures in fish. Reduced water temperature might be responsible for poor deposition of calcium materials (formation of opaque zone) during rainy season (May – August), while increased water temperature might influence increased deposition of calcium materials (formation of transference zone) during remaining seasons (September - April) in tropical fish. Decrease in water temperature is associated with reduced feeding activity of the fish, due to the poor plankton

Table 3 : Correlation between body length and body weight, otolith diameter with body length, weight, and otolith ring count with body length and weight in *S. balookee* (N = 134)

Correlation between	'r' value	Remarks
Body Length and Body weigh	0.94	Highly correlation
Otolith weight with Body length	0.67	Good correlation
Otolith weight with Body weight	0.73	Highly correlation
Otolith diameter with Body weight	0.55	Good correlation
Otolith diameter with Body length	0.55	Linear correlation
Otolith ring count with Body length	0.44	Moderate correlation
Otolith ring count with Body weight	0.47	Moderate correlation

* Correlation coefficient 'r' was calculated using Karl Pearson's method

population during these months in water bodies. Lombarte and Liconart (1993) suggested that otolith development occurs under dual regulation *i.e.*, genetic conditions regulate the form of otolith, while environmental conditions, mainly temperature (in carbonate-saturated water) regulate the quantity of material deposited during the formation of otolith. Further confirmation study is necessary for seasonal variation in deposition of calcium materials in otolith or other hard calcified materials in this fish.

Further, experimental studies have confirmed that the formation of growth marks are annual in many fish species including *Clarias gariepinus* (Wartenberg *et al.*, 2011), *O. niloticus* (Abdel-Hadi *et al.*, 2000) and *Puntius conchonius* (Bahuguna, 2013). Therefore the number of LAGs present in these structures directly depicte the age of individual fish. Presence of one to four LAGs suggests that this species might live for a maximum of five years. General trend in occurrence of growth rings in fish indicates that large number of smaller sized fish has lesser growth rings; whereas few larger sized fish had more growth rings in their calcified materials (Table 1). The frequency distribution of these growth rings in natural population further suggests that always a large number of smaller sized fish exhibited fewer growth rings and few large sized individuals possessed greater number of growth rings indicating the prevalence of a definite relationship between production and survival rate of this species.

Comparative study of otoliths vs scales and vertebrae showed that number of growth marks remained identical in otoliths and scales in 119 (88.81%) fish, but 15 (11.19%) fish showed one growth ring shorter in different age group fish. Scale age underestimated compared to otolith age in this species due to small sized transparent scale with un-cleared discontinuous growth rings. Khan *et al.* (2015) found that the annual rings present in scales in *H. molitrix* are inferior as compared to other calcified structures. On the other hand, vertebrae sections provided age readings that were very close to (99.25%) those from otoliths. Growth rings are very clear and distinct in the vertebrae sections due to wide periosteal layer. Similar results were reported in some fish, Polat *et al.* (2001) reported that vertebrae are most suitable and reliable aging structures in *Pleuronectes flesus luscus* as compared to scales and otoliths. Bahugun (2013) has proved that the vertebrae are the most reliable structure for the age determination of *P. conchonius*. Khan *et al.* (2015) has reported that vertebrae were reliable age estimating structure compared to scales and otoliths in *Mastacembelus armatus* and *Ompok pabda*. However, one (0.75%) fish showed one growth mark shorter in vertebrae as compared to otolith in the three year age group individual, this might be due to technical problems related to histological processing. Further, two individual's (1.49%) possessed one growth ring extra in the four year age groups as compared to ORC. Present investigation revealed that otoliths, as well as, vertebrae sections were most precise and reliable calcified materials for estimating age than scales in this species.

In order to study the longevity of fish in natural population, a random collection and large sample sizes (consisting of all the possible age groups) are advised to consider (Campana, 2001). The existing data on the longevity of tropical fish in nature reveal that *S. indica, N. notopterus, Channa marulius, C. gariepinus* and *Oreochromis mossambicus* live for 5–6 years (David and Pancharatna, 2003; Sudarashan and Kulkarni, 2013; Lad, *et al.*, 2014), while other fishes such as *C. mrigala*, and *C. catla*, are reported to live longer *i.e.*, 8 - 9 years.

In conclusion the presence of one to four annual rings in the three calcified structures in this fish population suggests that this fish live for a maximum of five years in nature. Formation of annual rings in the calcified materials of this fish appears to be influenced by scarcity of food; feeding intensity, breeding activities and reduced temperature which coincides with rainy season. Overall, results of the present study indicate that otoliths and vertebrae are most suitable aging materials as compared to those of scales in *S. balookee*.

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DETERMINATION OF AGE AND LONGEVITY OF ROAD MORTAL INDIAN COMMON TOAD *Duttaphrynus melanostictus* BY SKELETOCHRONOLOGY

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Age and longevity of road mortal Indian common toad, *Duttaphrynus melanostictus* ($N = 93$) inhabiting Sangli District (17°09′ N 74°45′ E) Western Maharashtra, Southern India was determined by skeletochronology. The Phalangeal Histology studies revealed no LAGs in 25.86% of the toads, whereas nine LAGs were observed in 1.07% followed by one LAG in 30.10%, two LAGs in 23.65%, three LAGs in 13.98%, and four LAGs in 5.38% of toads. Back Calculation indicated that the innermost peristeal LAG was partially eroded in nine (9.67%) individuals due to endosteal resorption. These results suggest that this species might live more than ten years in natural population.

Keywords: Amphibia; Anura; *Duttaphrynus melanostictus*; age; longevity; skeletochronology.

INTRODUCTION

Skeletochronology is most reliable and valid technique for studying age structure of rare, endangered and fossil specimens of amphibians and reptiles (Peabody, 1961; Castanet and Smirina, 1990; Castanet et al., 1993; Smirina, 1994; Guarino and Erismis, 2008; Sinsch, 2015). A majority of aging studies on amphibians are concentrated from temperate zones, where drastic fluctuations in the ambient temperature become a limiting factor for feeding activity and enforce the formation of seasonal bone growth for instance (Hemelaar, 1981, 1988; Smirina, 1983, 1994; Francillon et al., 1984; Castanet and Smirina, 1990; Cherry and Francillon-Vieillot, 1992; Wake and Castanet, 1995; Tejedo et al., 1997; Sinsch, 2015). Comparative studies on tropical amphibians are limited (Halliday and Verrell, 1988; Smirina, 1994; Sinsch, 2015). However, few skeletochronological studies are available on tropical anurans (Kulkarni and Pancharatna, 1996; Guarino et al., 1998; Pancharatna et al., 2000; Kumbar and Pancharatna, 2001a; Kumbar and Pancharatna, 2002; Pancharatna, 2002; Lai et al., 2005; Guarino and Erismis, 2008; Guarino and Erismis, 2014; Ashkavandi et al., 2012). The Indian common toad *D. melanostictus* is a widely distributed species in Taiwan, southern China, Hainan Island, southward through Southeast Asia to Indonesia, and also in westward to India and Sri Lanka (Shieh, 1993). This toad is found in plains as well as high altitudes up to 2500 m a.s.l. (Bahuguna et al., 2010). Lot of work has been carried out in the field of diversity, distribution (Khan, 2000), reproduction (Kanmadi and Saidapur, 1982; Saidapur, 1989; Saidapur and Girish, 2001; Ngo and Ngo, 2013), mating pattern, spawning behavior and sexual size dimorphism (Grampurohit and Radder, 2012) and mortality (Baskaran and Boominathan, 2010) of *D. melanostictus*. However, aging aspect of this toad has been poorly focused and is not well understood. In recent days, high rate of herpetofaunal mortality has been occurring on the roads due to heavy vehicular traffic in which amphibians were the most affected taxa (Das et al., 2007). Among the amphibians, highest rate of mortality has been recorded for the toad *D. melanostictus*, which might be due to the fact that they are slow to react to vehicles and also perhaps the drivers' ignorance (Basakaran and Boominathan, 2010). Bhupathy et al. (2011) have recorded more than 42 road mortalities in *D. melanostictus* out of 110 vertebrate species deaths during dry and wet seasons along the National Highway 220, which cuts the Western Ghats of India. High mortality of anuran species especially *D. melanostictus* have also been observed in Sangli and Karad road and other roads of Sangli district. The rate of mortality was very high at an early morning in the monsoon season from June to September. In the present investigation we are employing skeletochronological technique for determining the age and longevity for road mortal Indian common toad *D. melanostictus*.

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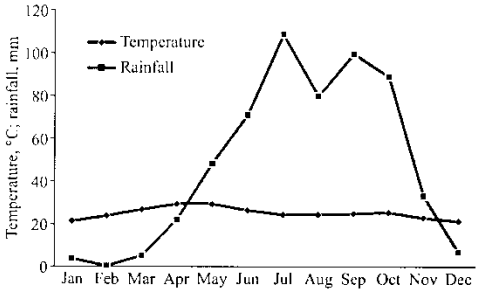

Fig. 1. Monthly variations in temperature and rainfall of Sangli District of 2014.

MATERIAL AND METHODS

Ninety three road mortal Indian common toad *D. melanostictus* [body weight: 5 600 g; body size (snout-vent length, SVL): 3.5 – 15 cm] were collected from the Sangli and Karad road (SH No. 75) at an early morning during rainy seasons (June 2013 to September 2015). Sangli district is situated in the southern part of Western Ghats of Maharashtra. It has a semi-arid climate with three seasons, a hot, dry summer from the middle of February to the middle of June; a monsoon, from the middle of June to late October; and a mild cool season, from early November to early February. The total rainfall is about 22 inches (580 mm). The type of vegetation is southern dry mixed deciduous and southern thorn forest (Kumbar and Patil, 2010). Collected toads were in different range of damages in which we picked up least damaged specimens and kept in plastic bags and brought to the laboratory where the body weight (to the nearest 1 g) and snout-vent length (SVL, measured to the nearest 1 cm using a thread) of each toad were recorded. The 4th toe of right or left hind limb (depending upon availability) of each toad were clipped and fixed in 10% formalin and numbered serially. Clipped toes were cleaned, washed in water for 1 – 2 h, de-mineralized in 5% nitric acid and processed for paraffin embedding. Sections of 8 µm thickness of the second phalanx of the 4th toe of each toad were cut on a Model GE-70 rotary microtome, stained with Harris hematoxylin and mounted in glycerin (Kumbar and Pancharatna, 2002). Mid-diaphyseal sections were chosen for observation under an Olympus CX41RF-16 compound microscope for the presence of growth rings, which were enumerated when present. Growth rings of each toad were counted separately by two individuals who were not aware of body weight and body size. The rate of endosteal resorption was assessed based on the comparison between the phalangeal sections at the same magnification from different individuals. The relationship between the body weight vs. SVL and number of growth marks vs. body weight and body size were assessed by drawing scatter plots and calculating correlation coefficients (r) by Karl Pearson's method (Steel and Torrie, 1980). The meteorological data provided in the present study were obtained from the Government of Maharashtra, Sangli District.

RESULTS

Monthly variation in temperature and rainfall of Sangli during the year 2014 is shown in Fig. 1. The mean temperature varied from 21.5 to 29.5°C, whereas the average rainfall was from 0.5 to 107.8 mm (Fig. 1). Cross-sections of phalanges of *D. melanostictus* showed growth rings, wherein each ring was composed of a faintly stained broader growth zone, and a darkly stained condensed chromophilic line, the LAG (Fig. 2). The growth rings were clear and distinct, and presence of either double or triple lines was not noticed in the individuals studied. Among the 93 toads examined, 24 toads (25.86%, SVL: 4.11 ± 2.08, Fig. 2*a*) showed no LAGs, 28 toads (30.10%; SVL: 7.36 ± 1.78, Fig. 2*b*) possessed one LAG each, 22 toads (23.65%; SVL: 7.12 ± 1.58, Fig. 2*c*) exhibited two LAGs, 13 toads (13.98%; SVL: 9.7 ± 1.64) showed three LAGs, five toads (5.38%; SVL: 9.48 ± 2.66) possessed four LAGs and one toad (1.07%; SVL: 15.2) exhibited nine LAGs in their phalangeal histology (Figs. 2*d*, 3). The distance between two LAGs was much variable in periosteal bone as the thickness of the growth layers progressively declined from the inner endosteal bone to outer periosteal bone layer, representing a decrease in bone growth in older individual (Fig. 2*d*). Histology of the distal phalanx of the smallest (body weight: 4 g, SVL: 3.5 cm) toad in our study showed a large marrow cavity in the center, circumference by a thin periosteal bone (Fig. 2*a*). When "back calculation" was made in order to estimate the loss of LAGs if any due to endosteal resorption, it was found that the innermost periosteal LAG was partially eroded in nine (9.67%) individuals, which was also confirmed by phalangeal histology (Fig. 2*c* and *d*). There was a high degree of positive correlation of the body weight and body size (r = 0.77; Fig. 4) and number of growth marks and body weight (r = 0.84, Fig. 5) and body size (r = 0.68, Fig. 6), respectively.

DISCUSSION

Skeletochronological observations on this toad reveal the presence of growth marks consisting of broader

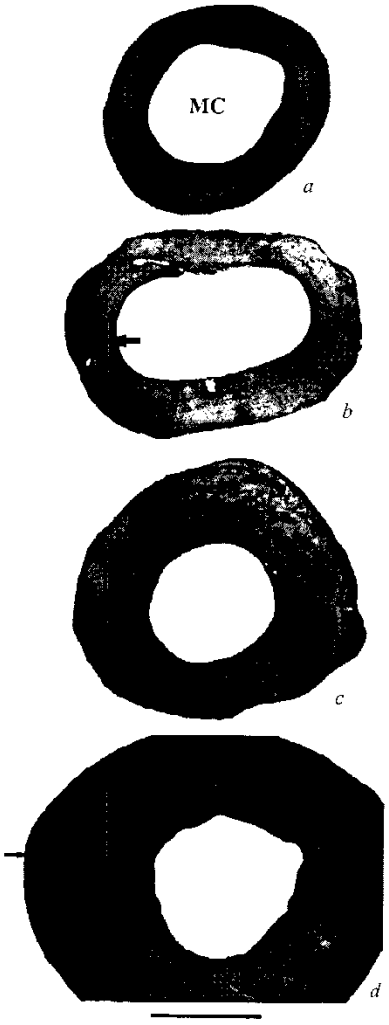

Fig. 2. Cross-sections of distal phalanges of *D. melanostictus*: *a*, phalangeal histology showing no LAG (SVL: 3.5 cm); *b*, with one LAG (SVL: 7.9 cm); *c*, two LAGs (SVL: 8.5 cm); *d*, with nine LAGs (SVL: 15.00 cm). Scale bar is 100 µm; arrows indicate LAGs (lines of arrested growths).

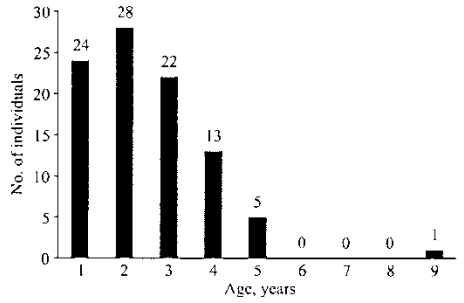

Fig. 3. Distribution of the toad *D. melanostictus* according to different age groups ($N = 93$).

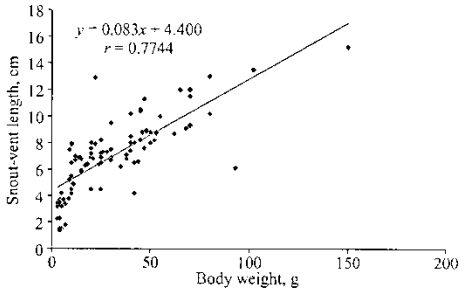

Fig. 4. Correlation ($r = 0.77$) of body size and body weight in *D. melanostictus*.

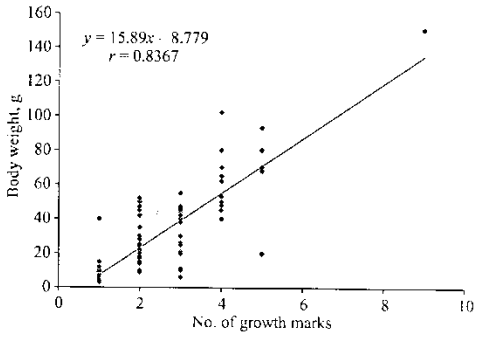

Fig. 5. Correlation ($r = 0.83$) of body weight and the number of growth marks in *D. melanostictus*.

growth zones and chromophilic LAGs in the cross-sections of phalanges (Fig. 2). This toad shows a clear-cut seasonality in body weight, fat-body weight, gametogenic and breeding activity (Kanamadi and Saidapur, 1982; Kanamadi et al., 1989; Saidapur, 1989) suggesting indirectly that bone growth is a cyclical phenomenon. Both body and fat-body weight reach their minimal values from June to August, which coincides with the onset

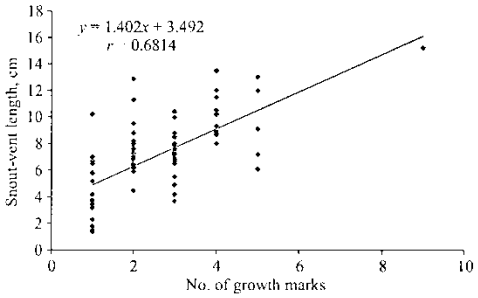

Fig. 6. Correlation ($r = 0.68$) of body size and number of growth marks in *D. melanostictus*.

of monsoon rains and breeding activity of the toad; from September onwards there is an increase in body and fat-body weight, and they attain their maximal values from December to April (Kanamadi and Saidapur, 1982; Kanamadi et al., 1989; Saidapur, 1989; Saidapur and Girish, 2000). Therefore, in this toad, the LAG(s) may be laid down from June to August when the body growth and food reserves almost ceased coinciding with the wet season of the year. From September onwards when body and fat-body weightes begin to restore, the next osteogenic cycle may begin. It is known that the rate of endosteal resorption severely influence skeletochronological interpretations in determining the age of amphibians (Halliday and Verrell, 1988; Castanet and Smirina, 1990; Smirina 1994). "Back calculation" in the present study revealed that the innermost periosteal LAG is partially eroded in nine (9.67%) large sized individuals due to endosteal resorption. There is no loss of complete LAG in these specimens; therefore, enumeration of periosteal LAGs directly depicted the age of individual toad. Furthermore, experimental studies have indicated that the formation of bone growth marks is annual in this toad; therefore, they can be regarded as year rings for estimating age (Kumbar and Pancharatna, 2004). In the present study out of 93 toads, 26% of toads were in the first year, 30% in second year, 24% in third year, 14% in fourth year, 5% toads in the fifth year and 1% in the tenth year of growth. Previous studies on age and longevity of southwest Indian species of anurans suggest that *Euphlyctis cyanophlyctis* [SVL = 3 – 7 cm], *Microhyla ornata* [SVL = 1 – 4 cm], *Limnonectes limnocharis* [SVL = 1.6 – 4.5 cm], *Hoplobatracus tigerinus* [SVL = = 6.5 – 17 cm] which have varied body size, live for a maximum of five to seven years (Pancharatna et al., 2000; Kumbar and Pancharatna 2001a; Pancharatna and Deshpande, 2003; Pancharatna and Kumbar, 2005). Our earlier skeletochronological record of Indian common toad *D. melanostictus* inhabiting Dharwad (15°17′ N 75°03′ E) southern India (Kumbar, 1999; Kumbar and Pancharatna, 2001a) live for maximum five years in contrast to the present investigation (17°09′ N 74°45′ E) whereas the oldest toad is found to be ten years. However, the toad *D. melanostictus* [SVL: 1.5 – 10.4 cm] inhabiting in Bhubaneswar (20°18′ N 85°50′ E), Eastern region of India live for maximum 12 years (Nayak et al., 2007). This difference in longevity in anurans may be related to the geographical position (altitudinal variations) of the respective regions because in the south, the annual variation in mean temperature normally does not exceed 10°C whereas the variation in temperature is around 25°C in the eastern region of India. This pattern suggests that lower temperature and reduced food availability at high altitude might increase the longevity and decrease the body size. Certainly, body size and longevity changes with altitude in most anuran individuals have been confirmed by many herpetologists (Miaud et al., 1999; Lu and Liang, 2006; Matthews and Miaud, 2007; Liao and Lu, 2010a). Therefore, comprehensive study is needed to confirm whether geographical variations, environmental condition, availability of food and genetic factor controlling body growth and longevity in tropical anurans.

There was a positive correlation between the body weight and body size and number of growth marks with body weight and body size, respectively. These results suggest that body weight and size may be a reliable criterion for determination of age in *D. melanostictus* unlike in other southern Indian species such as, *E. cyanophlyctis*, *M. ornata*, *H. tigerinus*, and *Polypedates maculatus* in which body size is correlated positively with the number of LAGs (Kulkarni and Pancharatna, 1996; Kumbar and Pancharatna, 2001a; Kumbar and Pancharatna, 2002; Pancharatna and Kumbar, 2005). Similarly, Indian green frog *E. hexadactylus*, *P. maculatus*, and *D. melanostictus* inhabiting eastern region of India showed high degree of positive correlation of body weight and body size (SVL) as well as body weight and body size with number of LAGs (Nayak et al., 2007; Nayak et al., 2008). By contrast, few species *Rana sylvatica* and *L. limnocharis* showed very poor correlation between body size and age (Leclair et al., 2000; Pancharatna and Deshpande, 2003). In conclusion, phalangeal skeletochronology can be used as a reliable technique for age determination in road mortal Indian common toad *D. melanostictus*.

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